

Drug/Inflammation Nutrient Transport Interaction in the Lactating Mother-Neonate Dyad

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ABSTRACT

This dissertation research involved investigations into possible drug-nutrient or disease-nutrient transport interactions in the nursing mother-neonate dyad. The overall hypothesis was that cefepime would inhibit L-carnitine transport at the lactating mammary gland and in developing neonates. Additionally, inflammation would alter energy substrate transporter expression in mammary tissue.

The first objective was to investigate the potential for drug-nutrient transport interactions at the lactating mammary gland. A continuous cefepime infusion to lactating rats reduced L-carnitine transfer into milk at early but not mid lactation. In conjunction with higher milk L-carnitine and cefepime concentrations and higher expression levels of Octn2, the data suggests cefepime competitively inhibited Octn2-mediated L-carnitine transport into milk.

The second objective was to assess the influence of lactation stage on milk-to-serum ratios (M/S) for an actively transported drug, cefepime, and its impact on the calculation of neonatal exposure indices. Higher cefepime M/S on day 4 lactation versus day 10 coupled with lower systemic clearance values for cefepime in postnatal day 4 versus day 10 pups resulted in >7-fold higher exposure index values at postnatal day 4. These data confirm the need to determine M/S at different lactation stages for actively transported drugs to avoid over- or underestimation of neonatal exposure risk.

The third objective was to examine a drug-nutrient transporter interaction in neonates. Cefepime administered twice daily according to different dosing schedules (postnatal days 1-4, 1-8, 8-11, 8-20 and 1-20) caused significant alterations in the ontogenesis of several mechanisms involved in the L-carnitine homeostasis. These alterations likely represented adaptive responses to cefepime inhibition of L-carnitine transport. Furthermore, these changes seemed to depend on duration and timing of exposure relative to postnatal maturation.

The fourth objective was to examine the effects of inflammatory stimuli on energy substrate transporter expression in mammary tissue. Inflammatory stimuli altered expression of glucose, fatty acid and L-carnitine transporters in mammary tissue *in vitro* and *in vivo*.

Collectively, this research provided experimental evidence for significant disease- or drug-nutrient transport interactions in the nursing mother-neonate dyad. Further research may identify a need for dietary modification during pharmacological management of disease in the nursing mother-neonate dyad.

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LIST OF ABBREVIATIONS

\bar{C}	Average concentration
ΔCt	Difference between Ct of target gene and Ct of β -actin
$^{\circ}C$	Degree of Celsius
μL	Microliter
μm	Micrometer
$\mu mole$	Micromole
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
Ag_2O	Silver (I) oxide
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
$ATB^{0,+}$	Amino acid transporter $B^{0,+}$
ATP	Adenosine triphosphate
AUC	Area-under-the-plasma-concentration-versus-time curve
Bbh	γ -Butyrobetaine hydroxylase
BSA	Bovine serum albumin
BW	Body weight
Cl	Clearance
Cl_o	Oral clearance
Cl_s	Systemic clearance
C_{milk}	Milk concentration
CN	Cyano
Cpt	L-Carnitine palmitoyl transferase

Cr	Creatine
CrP	Creatine phosphate
C _{serum}	Serum concentration
C _{SS,ave}	Average steady state plasma concentrations
Ct	Cycle threshold value
Ct2	L-Carnitine transporter 2
DMT	Divalent metal transporter
DTNB	5,5'-Dithio-bis (2-nitrobenzoic acid)
EDTA	Ethylenediamine tetracetic acid
EGTA	Ethylene glycol tetraacetic acid
EI _{conc}	Exposure index that relates neonatal plasma concentrations to maternal plasma concentrations
EIdose	Exposure index that relates neonatal dose as a percent of maternal dose
F	Bioavailability
FABP	Fatty acid binding protein
FAO	Food and Agriculture Organization
FAS	Ferrous ammonium sulfate
FAT	Fatty acid transport proteins
FATP	Fatty acid transport protein
FD	Fold difference
F ^{infant}	Infant bioavailability
F _{neonate}	Neonatal bioavailability
FPN	Ferroportin
g	Gram

GLM	general linear model
GLUT	Na ⁺ -independent sugar transporter
h	Hour
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt
HPLC	High performance liquid chromatography
HTML	3-hydroxy- N ⁶ -trimethyl-lysine
i.d.	Internal diameter
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IP	Intraperitoneal
IV	Intravenous
k	Elimination rate constant
KCl	Potassium chloride
KH ₂ PO ₄	Potassium phosphate monobasic
L	Liter
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M/P	Milk to plasma drug concentration
M/S	Milk to serum drug concentration
Mat	Maternal
MCF12A	Human mammary gland epithelia cell line 12A
MG	Mammary gland
mg	Milligram
MgCl ₂	Magnesium chloride

Min	Minute
mL	milliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
Na ₂ HPO ₄	Sodium phosphate dibasic
NaCl	Sodium chloride
Neo	Neonate
nm	nanometer
NS	No significance
NTCP	Sodium-taurocholate co-transporting polypeptide
o.d.	Outside diameter
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
OD	Optical density
PEPT	Di/tri-peptide transporter
pH	Potential of hydrogen
PMSF	Phenylmethanesulphonylfluoride
P-value	Probability of significance
PVDF	Polyvinylidene fluoride
RID	Relative infant dose
RIPA	Radioimmuno precipitation assay
RT-PCR	Reverse transcription polymerase chain reaction
RUF	Relative unit of fluorescence

SCD	Secondary L-carnitine deficiency
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGLT	Sodium dependent secondary active Na ⁺ /glucose transporters
SLC	Solute carrier
$t_{1/2}$	Elimination half-life
TAN	Total adenine nucleotides
TML	N ⁶ -trimethyl-lysine;
TNF- α	Tumor necrosis factor- α
UV	Ultraviolet
V	Voltage
V _d	Volume of distribution
V _{milk/τ}	Milk consumption rate
WHO	World Health Organization

1 Introduction

Mothers often take medication(s) for acute or chronic conditions while breastfeeding. While much attention in this area has focused on identifying the amount and mechanism of drug transfer into breast milk and the exposure risk to the nursing neonate, less attention has been paid to the potential effect of maternal disease and medications on nutrient transport in the mammary gland and the nursing neonate. Disease/drug-nutrient transporter interactions at the lactating mammary gland can affect the levels of specific nutrients and drugs in the breast milk (1). Such interactions can also take place in the nursing neonate when the drug is delivered via breast milk thus affecting nutrient availability and disposition in the neonate. Both these interactions can alter a neonate's nutritional status, which, in turn, can change the neonate's developing biochemical and physiological processes. Since the mammary gland and neonate experience rapid changes throughout the lactation period (2-4), such interactions may have different impacts depending on stage of lactation and neonatal development. Few studies have considered this possibility.

This dissertation work endeavors to advance our understanding of the possible interactions between maternal drug use/disease status and nutrient transport in the lactating mother-neonate dyad. These interactions are significant when they may change the breast milk composition of critical nutrients and nutrient status in the developing neonate. For this purpose, cefepime, an antibacterial drug, and L-carnitine, a conditionally essential nutrient, were used as a proof-of-concept for the ability of a drug to interfere with nutrient transport. Both cefepime and L-carnitine are substrates of the Organic Cation/Carnitine Transporters (OCTNs). The interaction between cefepime and L-carnitine at the transporter level may concomitantly increase cefepime but reduce L-carnitine levels in the breast milk and alter L-carnitine disposition in the developing neonate. Furthermore, the effects of an inflammatory stimulus on mammary gland nutrient transporter expression will identify the influence of disease on nutrient transport by the lactating mammary gland. The drug/disease-nutrient transport interaction will be assessed at different stages of lactation/neonatal development to identify the importance of considering the timing of the interaction relative to lactation stage and neonatal maturation.

2 Literature Review

2.1 The Risk-Benefit Ratio: Breastfeeding vs. Maternal Disease/Medication

Exclusive breastfeeding up to six months of age and continued breastfeeding for older infants are encouraged by the WHO (5). However, maternal medication usage to treat diseases during breastfeeding raises significant concerns, which can pose a difficult risk-benefit analysis. One major concern is neonatal exposure to the medication through breast milk. One must weigh the potential risk of neonatal exposure to the medication via breast milk against the benefit of breastfeeding for both mother and neonate. However, the risk of neonatal exposure also must be weighed against the risk of untreated maternal disease, which also may have negative effects on the neonatal growth and development and maternal health.

Under most circumstances, the benefits to both mother and neonate of continued breastfeeding with maternal medication use outweigh the risks (6). Several strategies and approaches have been proposed to minimize neonatal exposure and risk to medications via breast milk (7). However, these recommendations generally fail to consider the possible effects of maternal disease/medication use on breast milk nutrient composition and the potential consequences of such effects to the nursing neonate. Changes in nutrient availability to the developing neonate may pose a significant risk to the current and long-term health of the developing individual particularly when altered nutrition occurs during critical developmental stages. The potential for such interactions makes the risk-benefit analysis of maternal disease/medication use during breastfeeding more complex.

An appropriate risk-benefit analysis requires the identification of all potential risks and benefits to the mother-neonate dyad associated with treatment of maternal disease, the therapeutic measures recommended for maternal disease management, and the breastfeeding process itself. The ultimate goal of these risk-benefit assessments is to acquire a strategy that minimizes exposure risk and ensures optimal nutrient provision to the neonate, and provides the best therapeutic measures to the mother (8). However, these benefit-risk assessments are only achievable when sufficient scientific information and experienced clinical knowledge are available to the assessors. Currently, most research in this area focus on the drug present in the breast milk and the possible exposure risk to the nursing neonate. Unfortunately, the information on the risk of maternal diseases/medications on nutrient provision via breast milk, as well as neonatal exposure risk when the drug is present in the breast

milk at different postnatal ages for different durations of exposure is extremely limited. Research that addresses the impacts of diseases or drugs in nursing mother-neonate dyad on nutrient status during early life will add valuable information to the risk-benefit analysis, which can eventually help the health professional as well as the breastfeeding mother.

2.2 Breastfeeding: The Trends, Benefits and Problems

Women are returning to the practice of breastfeeding, a trend motivated principally by the multiple and unique advantages of human breast milk (8). Today, most healthy women initiate breastfeeding upon birth of their child (9-10), and in Saskatchewan, for example, the breastfeeding initiation rate is as high as 86% (11). The optimal duration of exclusive breastfeeding suggested by the World Health Organization (WHO) is six months based upon evidence gathered worldwide on infectious diseases morbidity and mortality (5).

Breastfeeding has a unique biological and psychological influence on the health of both mother and nursing neonate. Breastfeeding provides for the unique nutritional needs of the newborn during the transition from the *in utero* to *ex-utero* life and offers non-nutritional components that assist in adaptation to life outside the womb (12-13). These bioactive non-nutritional substances, not provided in infant formula, confer protection from bacterial and viral infections, which reduce mortality and morbidity in infants associated with respiratory and gastrointestinal diseases, and aid in the growth and development of the newborn (14-17). Epidemiological data also indicate that breastfed infants have lower rates of obesity into childhood and adolescence compared to formula-fed infants (18) and improved cognitive functions (13).

For the nursing mother, breastfeeding promotes the bonding between the mother and baby. Breastfeeding is also associated with decreased incidence of cancer and osteoporosis (19). It also helps to prevent post-partum hemorrhage (20). Breastfeeding mothers have less risk for anemia and long term obesity compared to bottle-feeding mothers (21-22). In diabetic mothers, breastfeeding helps reduce insulin requirements (23).

In addition to the health advantages, the economic benefits of breastfeeding to both parents and the health system are felt through reductions in direct and indirect

costs (24). Direct savings can be achieved through breastfeeding since there is little or no need for infant formula. The cost for purchasing infant formula can add up to \$1,200–\$1,500 or more for a baby's first year(25). Direct benefits also arise from reductions in physician visits, hospital stays, and other expenditures related to the treatment of illness (24, 26). Indirect costs may appear in the form of lost wages due to breastfeeding mothers attending a sick child.

Occasionally, circumstances arise when breastfeeding can pose a risk to the neonate. Presence of drugs in milk has raised significant concerns when mothers decide to breastfeed (8). According to current guidances, infant exposure doses that constitute less than 10% of the mother's medication dose on a body weight basis are arbitrarily considered to be safe (27-28). This guideline is supported by the general lack of overt adverse events reported in nursing infants when breastfeeding mothers take medications (29). However, long-term adverse outcomes in neonates following such exposures are not available. In addition, the neonate's immature renal function and hepatic xenobiotic metabolism capacity does raise some concerns about the safety of neonatal exposures to drugs in breast milk (30-31). Compared to the adult, neonates have less capacity to eliminate drugs from the body due to the underdeveloped elimination pathways. For example, expressions of several members in cytochrome P450 family of drug metabolizing enzymes increase during postnatal development (32). Thus, drugs delivered to the nursing neonate can accumulate in the body and eventually lead to toxic levels and adverse effects.

Another concern is the ability of drugs to affect nutrient availability and disposition in the neonate and nutrient composition of the breast milk. Since early life nutrition is critical to the well-being of the neonate, drug induced nutrient alterations may result in detrimental consequences either acutely or more subtly by predisposing neonates to adult onset chronic disease (33). Moreover, the dynamic changes in the mammary gland function during the lactation stage (34) make the safety prediction based on maternal dose more difficult(35). During the lactation period, the synthesis and secretory functions of the mammary epithelial cells as well as the milk content experience rapid changes. Drugs delivered into the mammary gland have the ability to cross the mammary gland barrier via the nutrient secretion pathways, thereby inhibiting these transport mechanisms (29). The change of the mammary gland nutrient transport function with lactation stage can influence the

amount of drug excretion into the breast milk and the degree of inhibition of nutrient transport. Such phenomena can make risk assessment of drug exposure during lactation difficult.

2.3 Breast Milk and Neonatal Nutrition

The postnatal period is marked by rapid growth and development of the neonate. This rapid growth places high demands on the supply of various nutrients from the breast milk (12). Immediately after birth, the maternal umbilical supply of substrates stops abruptly, and the newborn must adapt to an *ex utero* supply of nutrients, namely the breast milk. The nutrients delivered to the neonate must ensure continuous growth and maturation by assuring the provision of adequate Calories, appropriate balance in fluid homeostasis, as well as essential macro- and micronutrients and other non-nutrient components. Studies have shown that neonatal plasma concentrations of various nutrients during the early postnatal period correlate with the availability of nutrients from breast milk (36-37).

2.3.1 Breast Milk as a Source of Nutrition to the Nursing Neonate

Human lactation evolved in attempts to provide adequate and unique nourishment to the nursing neonate regardless of circumstance and environment (38). The composition of human milk is very complex with thousands of constituents that include proteins, carbohydrates, lipids, vitamins, minerals, nonnutrient bioactive substances, as well as cells (39). A number of these constituents can be generally categorized into macronutrient and micronutrients based on their physical and physiologic properties.

Since “macro” means large, macronutrients are nutrients that are needed in large amounts. Macronutrients contained in human milk are lipids, proteins and nonprotein nitrogen, and carbohydrates (39). Lipids constitute 3% to 5% of human breast milk (40), the second highest constituent of breast milk, with water being the principal component. The lipids in the breast milk vitally impact neonatal growth and development by providing a dense energy source and essential fatty acids such as docosahexaenoic acid (DHA) (41). The majority of milk lipids is derived from maternal circulation and storage with a small portion synthesized *de novo* by the mammary gland (41). Therefore, maternal dietary lipid intake can have a profound

influence on the milk lipid content. Interestingly, lipid composition changes with lactation stage and total fat content increases with duration of lactation (42-43).

Milk proteins are also important macronutrients of breast milk. Proteins account for 0.9% of human milk content (44). The major proteins include casein, various immunoglobulins and a variety of glycoproteins. Milk proteins are mostly synthesized in the mammary gland with the minor portion supplied by the maternal circulation (45). Milk proteins provide essential amino acids needed for neonatal growth and development (46). Immunoglobulins protect the nursing neonate against certain pathogens (46). Both the quantity and quality of milk proteins changes with the stage and duration of lactation. In general, protein content decreases during the course of lactation (46). The nonprotein nitrogen in breast milk consists of compounds including free amino acids, nucleotides, and nucleic acids (47). Though various lipids are the major energy-yielding substrates in breast milk, carbohydrates also provide energy for the newborn (39), and are essential for central nervous system kidney, brain, muscle (including the heart) function.

Micronutrients are nutrients needed only in small amounts. These nutrients are the “magic wands” that enable the body to produce enzymes, hormones and other substances essential for proper growth and development (48). The main micronutrients contained in breast milk are vitamins, minerals such as calcium, magnesium and phosphorus, and trace minerals including iron, copper, and zinc (39). These micronutrients can modify hormones, growth factors and cell signaling pathways, with subsequent effects on development during early life (49). Although these compounds are required only in small amounts, severe consequences ensue in their absence. Micronutrient imbalance or deficiency during gestation or postnatal life can have detrimental effects on the individual (49). Often the effects of micronutrient deficiencies go unnoticed despite their insidious effects. As a result, micronutrient deficiencies have been labeled as the “hidden hunger” (50-51).

Conditionally essential nutrients are nutrients that can be synthesized in a healthy individual in sufficient quantities to meet physiological requirements. However, during certain stages of the life cycle, such as newborns or advanced age, or under certain conditions (e.g. stress, disease, intoxication, etc), such nutrients become essential dietary components because their requirements may exceed an individual's capacity to synthesize them (52). Conditionally essential nutrients with bioactive functions in human milk include nucleotides, L-carnitine and choline. For

example, L-carnitine can be synthesized in the human liver, but insufficient amounts may be produced in neonates, adolescents and adults under certain physiological conditions such as recurrent pulmonary infections (53-55). Thus, exogenous sources of L-carnitine become necessary to maintain normal L-carnitine levels in the body.

Compared to infant formulas, human breast milk is unique and remarkably dynamic. Generally, the quantitative levels of nutrients in infant formula are kept within a homogenous range. However, the volume and composition of breast milk changes depending on the stage of lactation. For example, the fat and protein levels in human breast milk change considerably in the first several weeks of lactation (42-43). The dynamic change in breast milk has been associated with the changing demands of the developing neonate (56). Another unique aspect of human breast milk is its interindividual variability. Breast milk composition varies widely among women, particularly women from different ethnic and nutritional backgrounds. Most recommendations for infant formulations are based on data obtained from mature human milk of mothers with Caucasian ethnicity and typically western diets (57). These recommendations ignore the factor that both genetic and maternal dietary choice can affect breast milk composition (58). Infant formula manufactured according to these recommendations may not be the optimal nutrient source for the developing infants. Consequently, it may lead to unfavorable development in the nursing infants which may eventually lead to health problems in later life (59). Furthermore, various environmental and maternal conditions can alter breast milk composition, which can result in significant intraindividual variability compared to infant formula (60-61).

2.3.2 Early Life Nutritional Programming

Nutrients during critical windows of development in fetal, neonatal, infant and child development have important metabolic programming effects that may influence both the present and future health of the developing individual. Nutrition-induced alterations in biochemical and physiological processes during these vulnerable periods of susceptibility cause permanent adaptations in later life (59). A mild nutritional alteration that does not substantially affect development during early life stages may prepare the developing individual's physiology to improve survival in the predicted adult environment through appropriate phenotypic modifications (62). Alternatively, such adaptations may pose a serious risk for chronic disease when this

“predicted” adult environment is altered unexpectedly (63). Predisposition to the metabolic syndrome, which progresses to cardiovascular disease and diabetes, has been associated with inappropriate fetal or neonatal nutrition (64).

The association between prenatal nutrition and chronic disease development in adulthood has been extensively investigated (65). The “fetal origins hypothesis” proposed by Barker et al. well describes the association between adverse intrauterine conditions and diseases such as hypertension and insulin resistance in the adulthood (66-67). However, early postnatal life also represents a critical developmental stage during which altered nutrition can ‘program’ an individual’s future health. Epidemiological studies support the importance of early postnatal nutrition on the susceptibility to chronic disease including hypertension, obesity and type 2 diabetes (19, 68-69). Breast milk remains the preferred source of optimal nutrition in postnatal life and many advocate breast milk as superior to infant formula. For example, a reduced risk for high blood pressure during adult life is associated with the low sodium content and high levels of long chain polyunsaturated fatty acids in breast milk compared to infant formula (70-71).

Most studies examining the influence of postnatal nutrition largely focus on the consequence of maternal dietary manipulations in macronutrient intakes such as fat and protein. For example, rat pups exposed to maternal undernutrition (protein-free diet) during the sucking period resulted in permanent changes in insulin and glucocorticoid secretion, which may eventually predispose these pups to chronic disease in adult life due to impairment in the inflammatory response (63). However, few studies have investigated the consequence of micronutrient alterations and its long term consequences (49). Vitamins and minerals play crucial roles in metabolism and growth. Sufficient amounts are critical for the healthy development of the embryo, fetus and neonate (49). Certain micronutrient deficiencies such as zinc during pregnancy can result in fetal malformation and other defects (72). The effects of micronutrient alterations during early developmental stages become more problematic when interactions occur between these micronutrients and maternal drug/disease (73). Certain diseases and therapeutic drug usage during pregnancy or lactation may alter micronutrient status in the maternal circulation and their transfer across the placental or mammary epithelial barriers, which can lead to changes in fetal or neonatal micronutrient status. Such changes may have long term health effects.

2.3.3 Transport of Milk Constituents in the Mammary Gland

Optimal infant nutrition is crucial for neonatal growth and development. Early accelerated growth during critical developmental windows may induce obesity and reduce life span in rats (74). Malnutrition, including both excess or undernutrition, during neonatal development may have implications on later health. This suggests the various nutrient levels in the breast milk need to match neonatal requirements at each stage of development. A variety of mechanisms in the lactating mammary gland ensure appropriate nutrient levels during each stage of lactation.

Five major pathways exist in the mammary gland by which compounds are secreted into the breast milk (Figure 2.1). These include: 1) Exocytosis; 2) Transmembrane transporters; 3) Milk fat globule secretion; 4) Paracellular route, and 5) Transcytosis (75-76). During lactation, the paracellular pathway in the mammary gland is limited due to the formation of tight junctions, and transcellular transport pathways become the major routes of milk constituent secretion (77-78). Aqueous solutes including the major milk proteins are transported into breast milk through the exocytotic routes (79-80). Lipids and lipid-associated proteins are secreted via milk fat globule secretion, which is unique to the mammary epithelial cells (81). Most of the macromolecular substances such as immunoglobulins are transferred into milk via transcytosis (82). In addition, various ions and small molecules such as glucose and amino acids can be transferred from maternal circulation to the milk via specific transporters at the cellular, Golgi or secretory membranes (75-76).

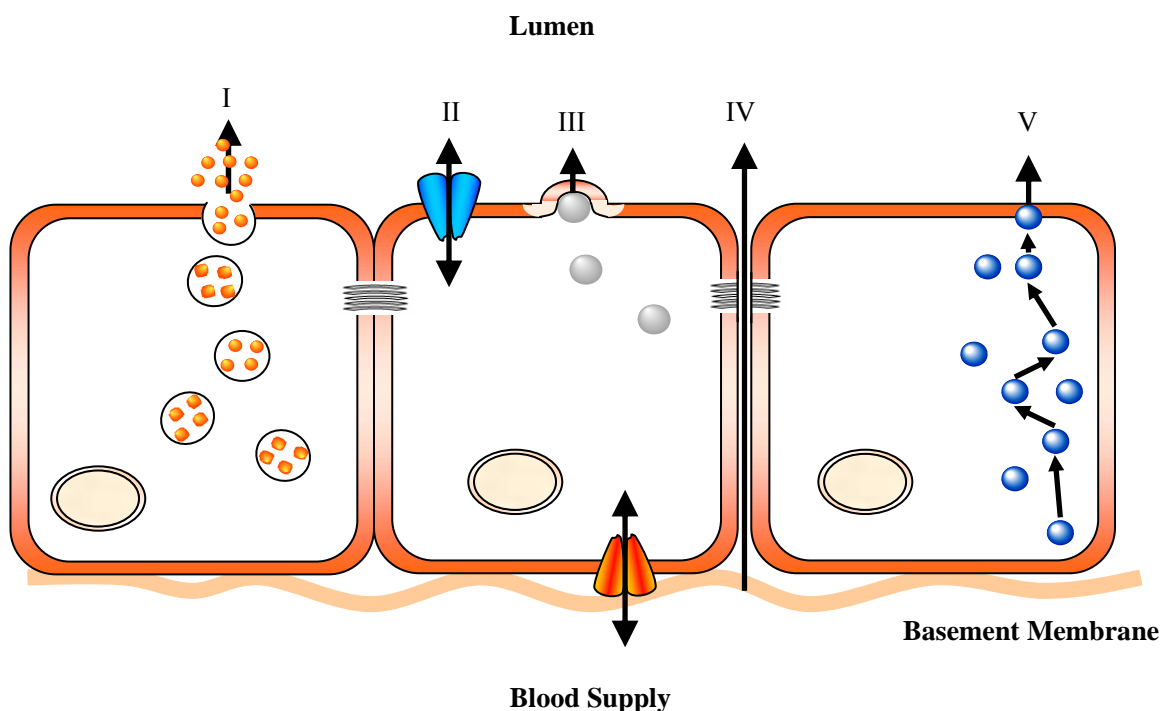


Figure 2.1. The known major pathways exist in the mammary gland by which compounds are secreted into the breast milk. I Exocytosis; II Transmembrane transporters; III Milk fat globule secretion; IV Paracellular route, and V Transcytosis. (Adapted from McManaman and Neville (75)).

2.3.3.1 Nutrient Transporters in the Mammary Gland

Nutrient transporters play crucial roles in movement of nutrients across polarized epithelial barriers, and cellular availability and disposition of nutrients in the body. In the mammary gland, nutrient transporters are responsible for transfer into and the milk composition of a variety of nutrients. As well, the same nutrient transporters are responsible for nutrient absorption, distribution and elimination in the nursing neonate.

To date, numerous transporters have been identified in the lactating mammary gland (76, 83-84). Various members of the Solute Carrier (SLC) and the ATP-Binding Cassette (ABC) transporter families are expressed during lactation. Such transporters may play important roles in maintenance of mammary gland function as well as milk secretion. With the onset of lactation, mammary gland epithelial cells have a high demand for precursor molecules such as glucose and fatty acids to facilitate production of high amounts of lactose and lactose-derived oligosaccharides as well as triglycerides in the breast milk (84-85). Mammals express two different

types of glucose transporters: facilitative glucose transporters (GLUTs) and sodium dependent secondary active Na^+ /glucose transporters (SGLTs) (75). Members from both GLUT and SGLT families are expressed in lactating mammary epithelial cells (84-85). Fatty acid uptake pathways include fatty acid binding proteins (FABPs) located in the plasma membrane, fatty acid translocators and fatty acid transport proteins (FAT) (86). Therefore, glucose and fatty acid transporters in the mammary gland play critical roles in milk production and mammary epithelial cell function (87). Interestingly, the transfer of glucose and galactose into mammary epithelial cells is the rate-limiting step in the milk lactose biosynthesis pathway (88).

In addition, other macronutrients needed for milk production, such as amino acids and peptides, are delivered to the mammary gland through various amino acid transporters and the oligopeptide (PEPT) transporters, respectively (89). An increasing amount of information is becoming available on micronutrient transport in the mammary gland (90). Essential metals such as zinc, iron, and copper are actively transported into milk via specific transporter systems (91-92). Other vitamins, minerals and hormones also possess specific transporter systems for their transfer into milk (93-94).

Nutrient transporter expression in the lactating mammary gland does not seem to remain static through lactation, but rather expression levels change with lactation stage (95). For example, the mRNA expression level of the amino acid transporter $\text{B}^{0,+}$ ($\text{ATB}^{0,+}$) decreases from early to peak lactation stages in sows (96). GLUT1 expression increased until the mid-lactation in mice (97-98). The expression levels of the two iron transporters, divalent metal transporter 1 (DMT1) and ferroportin1 (FPN1), in rat mammary epithelial cells decreased throughout the course of lactation (99) and this decrease correlated with the normal decline in milk iron during lactation (100). Also, the normal decline in milk copper concentration is attributed to the decrease in expression of the copper specific transporter, Atp7B, in the rat (101). Such studies suggest that changes in transporter expression may reflect alterations in milk composition of such nutrients through lactation.

Transporters responsible for nutrient transfer across the mammary epithelial barrier also can transfer xenobiotics (drugs, toxicants) into breast milk. A variety of drugs are secreted into breast milk via these nutrient transporters. Cationic drugs have a tendency to accumulate in breast milk via the organic cation transporters, which occasionally leads to toxicity in the nursing neonate (102-103). *In vitro* studies

suggest certain cationic drugs can inhibit nutrient transport across the mammary epithelium. Both verapamil and desipramine caused complete inhibition of L-carnitine uptake in a normal mammary gland epithelial cell line (MCF12A) via inhibition of hOCTN2 and ATB^{0,+}, and decreased glycine uptake through inhibition of ATB^{0,+} (104). Such cationic drug-nutrient transporter interactions demonstrated *in vitro* may have the potential to change breast milk nutrient composition *in vivo*. Furthermore, given the changing expression levels of nutrient transporters with stage of lactation, the magnitude and significance of such interactions can vary depending on lactation stage. When the affected nutrients are obligatory for neonatal development, the drug-nutrient transporter interaction may pose a detrimental risk to the nursing neonate.

2.4 Effects of Drug/Disease on Mammary Gland Function during Lactation

The mammary gland undergoes significant physiological and biochemical adaptations during pregnancy and lactation to ensure neonatal survival. Any significant disruption of mammary gland function during lactation can cause alterations in both milk volume and composition, which can be unfavorable to neonatal development. Mothers with illness or disease often use drugs for treatment of their condition during breastfeeding. Both disease and medications taken by the breastfeeding mother have the potential to impact mammary gland function and, therefore, cause changes in breast milk.

2.4.1 Mammary Gland Physiology and Biochemical Activities during Lactation

The Class “Mammalia” was named for species that use a mammary gland to feed their offspring (105). The mammary gland provides a vital survival and evolutionary advantage to mammalian species (106-107). The mammary gland undergoes development predominantly after birth unlike other organs of the mammalian system. The most pronounced morphogenesis of the mammary gland occurs during pregnancy and lactation. During pregnancy, a variety of changes including extensive growth of duct and alveoli, proliferation of mammary glandular tissue, mammary epithelial cell differentiation, and vascularization occur preparing the mammary gland for its specialized secretory functions in lactation (17, 108). The functional development of this organ is fundamentally dependent upon hormones and

growth factors such as estrogen, progesterone, and prolactin (109).

The basic milk production units in the mammary gland are the alveoli, which are mainly constructed of epithelial cells. These epithelial cells contribute significantly to the production and transport of milk constituents into the alveolar lumen (110). Each alveolus unit is surrounded by a network of myoepithelial cells, contractile cells that force the milk from the alveoli into the ductile system. Other types of cells found in the mammary gland include fibroblasts, adipocytes and immune cells (111). With the onset of milk secretion (lactogenesis), the highly differentiated mammary epithelial cells undergo a further rapid series of changes. The tight junctions between the epithelial cells close in the first week post-partum to form a highly impermeable barrier between milk and interstitial fluid (78). These tight junctions polarize the mammary epithelium into distinct apical and basolateral cell surfaces and prohibit the paracellular movement of the major milk macro- and micronutrients between the extracellular spaces to the lumen of the mammary alveoli (78). In addition, the synthetic activities of the mammary alveolar cells increase with advancing lactation leading to changes in milk volume and composition (3).

During lactation, mammary epithelial metabolic activities also increase dramatically to meet the requirement for milk synthesis and secretion in the mammary gland. For example, high proportions of substrates including glucose and fatty acids are circulated into the mammary gland to synthesize the major milk components, lactose and lipids(112). In lactating rats, mammary epithelial cells consume 80-90% of the glucose obtained from the maternal circulation (113). Glucose contributes significantly to the energy requirement for milk production in lactating mammary epithelial cells and is the primary precursor for lactose synthesis (113). Since mammary epithelial cells cannot synthesize glucose *de novo* (114), the transport of glucose from the maternal circulation into the mammary epithelium becomes crucial and this transport is mediated principally by the GLUTs.

Although glucose contributes significantly to the energy requirement for milk production in the mammary epithelial cells, fatty acids provide the remaining energy source. Fatty acids are also precursors for triglycerides in the breast milk (115). The availability of fatty acids to the mammary gland depends on both *de novo* biosynthesis in the mammary epithelial cell and uptake from maternal circulation (116). Fatty acid uptake pathways include fatty acid binding proteins located in the plasma membrane, fatty acid translocators and fatty acid transport proteins (86).

Failure to make these biochemical adjustments in the mammary gland to meet the requirements of mammary gland function may lead to unfavorable consequences with reductions in both mammary gland synthetic and secretion activities.

2.4.2 Effects of Maternal Medication on Mammary Gland Function

Neonates have a narrow tolerance range for changes in nutrient supply from the breast milk (56). Maternal medications that result in inadequate or excessive supply of nutrients in the breast milk may lead to short- or long-term disadvantages in neonatal development. Therefore, information on how maternal medications may affect breast milk quantity and quality is critical.

In theory, xenobiotics have the potential of disrupting the development or maturation of the mammary secretory epithelium (56, 117). Xenobiotics may affect milk volume and composition through direct action on the mammary gland tissues (118-119), or indirectly through systemic changes in the lactating mother (e.g. alteration of the hormonal milieu necessary for milk secretion; alteration of availability of substrates of synthesis/secretion of milk constituents) (117).

2.4.2.1 Direct Effects of Maternal Medication on the Lactating Mammary Gland

Much evidence is available regarding the effects of drugs on milk quantity and quality through direct effects on mammary gland development (120-122). The mammary gland has a complicated functional architecture to ensure its critical secretion functions. Any interruption in the formation of this architecture may result in unfavorable consequences in milk secretion. Steroids and other growth stimulating drugs are known to alter mammary gland growth and development (118).

The tight junction formed during lactation ensures the transepithelial gradient necessary for milk secretion. Drugs such as 5-hydroxytryptophan have the potential to compromise tight junction integrity, which leads to marked changes in breast milk composition (121). Other drugs may directly affect mammary gland biosynthetic functions. For instance, the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, inhibits milk protein synthesis, including casein and α -lactalbumin, in a mammary gland cell culture system (120, 122).

2.4.2.2 Indirect Effects of Maternal Medication on Milk Production

Maternal medications can indirectly affect mammary gland function via interference with the hormones of lactation or with the “nutrikinetic” processes involving biosynthesis and transport of milk substrates.

2.4.2.2.1 Effects of Maternal Drugs on Hormonal Milieu Supporting Lactation

A small group of medications have an adverse effect on milk production by interference with the two major hormones that support lactation: prolactin and oxytocin. Drugs with androgenic or estrogenic properties and vitamin B₆ can reduce prolactin levels and thereby decrease milk production (7). Alcohol is a potent inhibitor of oxytocin release, which consequently results in decreased milk production (123). Since infant weight gain and development are directly associated with milk production, the potential problems associated with reduced milk supply are of importance (14). Drugs can also change the composition of milk. In addition to its impact on milk production, alcohol can decrease lactose and increase the lipid content of breast milk (14). Alterations in prolactin levels also impact milk zinc secretion via affects on the zinc transporters (90).

2.4.2.2.2 Drug-Nutrikinetic Interactions Affecting Milk Secretion

Alternatively, drugs can interfere with maternal nutrient availability and other “nutrikinetic” processes in the mother thereby reducing the availability of such nutrients for the breast milk (124-125). Drugs can: 1) Directly inhibit the biosynthesis of specific nutrients. For example, mildronate treatment decreased L-carnitine levels in rat milk by inhibition of gamma-butyrobetaine hydroxylase (γ -BBH), the enzyme catalyzing the last step of L-carnitine biosynthesis (125). 2) Inhibit gastrointestinal nutrient absorption. For example, cholestyramine, bile acid sequestrant used to treat hypercholesterolemia reduced the absorption of the fat-soluble vitamins, A, D, E and K, resulting in a deficiency of these vitamins in pregnant mothers and their fetuses (126-127). 3) Interfere with nutrient metabolism in the maternal body. Vitamin B₆ levels are lower in the breast milk from mothers who take oral contraceptive agents compared to nonusers (128). Oral contraceptive agents interfere with vitamin B₆ metabolism in the body, thereby reducing maternal levels (129) and levels in the breast milk. 4) Increase nutrient elimination. For example, excessive aspirin doses can cause increased urinary folate excretion

following displacement from plasma protein binding sites(130). The interaction between aspirin and folate may eventually lead to maternal folate deficiency and, thus, decreased folate concentration in the milk (131). 5) Compete with nutrients for the transporters in the maternal body to cause nutrient excess or deficiency in the mother depending on transporter function. Limited information is available for the potential risk of drug-nutrient transporter interactions on breast milk nutrient levels. However, drug-nutrient interactions at the transporter level have been identified *in vitro* (104). As the sole source of nutrition for the neonate, alteration in the breast milk composition of various nutrients through indirect or direct drug effects may have a critical impact on neonatal health and development (132).

2.4.3 Effects of Maternal Disease on Mammary Gland Function

Maternal medication use occurs when a need exists for the treatment of maternal illness or disease. Generally, mothers with chronic disease are encouraged to breastfeed (14). However, some maternal diseases are contraindicated for breastfeeding or can be associated with long-term adverse consequences for the nursing neonate (14). Epidemiologic studies indicate an association between maternal diabetes and adult obesity and glucose intolerance in the breastfed offspring (133). Breastfed neonates from mothers with asthma have a higher risk for asthma due to the presence of immunologically active substances in the breast milk (134). Moreover, literature reported several cases of vitamin B₁₂ deficiency and other related symptoms in breastfed infants of mothers who had low vitamin B₁₂ levels due to latent pernicious anaemia secondary to hypothyroidism (135-136).

2.4.3.1 Inflammatory Conditions of the Mammary Gland and Breast Milk

Mastitis, an inflammatory response in mammary tissue often caused by bacterial infections, is a common condition observed during the first 6 months of breastfeeding (137). Incidence rates in breastfeeding mothers vary 2 to 33% in the United States (138). Mothers with mastitis are usually encouraged to continue breastfeeding; however, breastfeeding-associated inflammatory breast diseases contribute most to an early cessation in breastfeeding (139). The most common infective agents responsible for mastitis are Escherichia coli (gram-negative) and Staphylococcus aureus (gram positive). Infection induced by E. coli is usually acute,

which usually resolves within a few days (140). However, mastitis caused by *S. aureus* often results in a chronic low-grade infection in the mothers (141).

Mastitis disrupts mammary gland function, which can lead to changes in both the quantity and quality of breast milk. Inflammation of the mammary gland can result in physical damage to the mammary epithelium that decreases its synthetic and secretory capacity (142) and enhances its permeability (78, 143). Acute mastitis can also cause an opening of tight junctions (78, 143), which would lead to enhanced permeability of mammary epithelium. Leaky tight junctions are implicated in the lower lactose and a higher sodium levels in mastitic breast milk (144). Mastitis also leads to an increase in milk proteins, such as albumin and immunoglobulins, and decreases in caseins (142). Inflammation and the offending pathogens can alter the metabolic activity of mammary epithelial cells. This could result in reductions in α -lactalbumin and β -lactoglobulin often observed during mastitis (142) and possibly other breast milk constituents. Moreover, these mastitic pathogens can produce enzymes that have the capacity to decompose milk constituents. For instance, proteinases produced by the mastitic pathogens can degrade milk casein (142). Consequently, through physical damage and alterations in metabolic activity of the mammary epithelium, inflammatory disease of the mammary gland can produce significant changes in breast milk composition and volume.

2.4.3.2 Effects of Inflammation on Transporter Function

Inflammatory stimuli can induce immunological processes in the mammary gland. This is a highly studied area of research. However, the ability of inflammatory states to alter nutrient transport in the mammary epithelium has received limited attention. *In vitro* studies have identified the ability of inflammatory cytokines to alter transporter expression in cell culture (145-146). In an intestinal cell culture system, interferon- γ (IFN- γ) increased di/tri-peptide transporter (hPEPT1) expression and dipeptide transport (145). In primary hepatocytes, the proinflammatory cytokines TNF- α and IL-6 decreased sodium-taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP) 1B1 protein expression and transport activities (146). Numerous studies have also identified inflammation-mediated changes in transporter expression *in vivo*. For example, intestinal fructose transporter GLUT5 expression and activity decreased during intestinal inflammation across various species (147). LPS

(Lipopolysaccharide, a gram-negative bacterial cell wall component) challenged mice demonstrated marked downregulation of renal tubular glucose transporters SGLT2, SGLT3 and GLUT2 with concomitant decreases in plasma glucose concentrations (148). Moreover, the effects of inflammation on various drug transporters and the pharmacokinetic consequences of altered drug transporter expression have been extensively studied (149). Clearly, changes in nutrient transporter expression with inflammation can alter nutrient absorption, distribution and elimination, which, in the lactating mother, may affect nutrient availability to the mammary gland. Although most of the data is obtained from tissue or cell lines other than mammary gland, such data suggest that inflammatory stimuli may alter transporter expression and activity in the lactating mammary epithelium and influence the composition of breast milk.

2.5 Risk of Drug Exposure via the Breast Milk

Any medication taken by the nursing mother has the potential to cross the mammary epithelial barrier into milk and be passed on to the nursing neonate (150-152). For instance, all psychotropic medications taken by nursing mothers have been detected in their milk and measurable quantities have been identified in their nursing neonates (153). However, many medications are not found at detectable levels in the breast milk (7) and most medications taken by breastfeeding women do not cause overt adverse events in the nursing neonate (29). Nevertheless, some drugs accumulate to significant levels in breast milk (154). Even low level exposures cause some concern as neonates can be more susceptible to the same substances compared to the adult (155). Furthermore, immature elimination mechanisms in the neonate can result in significant drug accumulation even following low exposures via the breast milk (30-31, 155). Thus, drugs present in breast milk at low levels may still pose a significant exposure risk to nursing neonates (30-31).

2.5.1 Relative Infant Dose and Infant Exposure Index

The major factors that determine neonatal exposure to drugs via the breast milk are the amount of drug present in the breast milk and the capacity of the neonate to eliminate the drug (as measured by systemic clearance) (8). A measure of drug distribution into breast milk is the milk-to-plasma drug concentration ratio (M/P ratio) (156). The M/P ratio has been widely used as the index for neonatal drug exposure

via the breast milk (157). However, as an exposure index, it offers little information regarding the actual exposure of the neonate. Currently, the relative infant dose is the most commonly used exposure index for assessing drug safety during breastfeeding. RID is the percentage of absolute infant dose relative to the maternal dose corrected for body weight (Equation 2.1). The absolute infant dose is calculated from the average maternal plasma drug concentration ($C_{ave,maternal,plasma}$), the M/P ratio and the milk consumption rate (V_{milk}/τ). Drugs with RID less than 10% of the maternal dose are generally considered as safe(27).

$$\begin{aligned}
 RID &= \frac{\text{Absolute Infant Dose (mg/kg/day)}}{\text{Maternal Dose (mg/kg/day)}} \times 100\% \\
 &= \frac{F_{mat} \times C_{SS,mat} \times (M/P) \times (V_{milk}/\tau)_{infant}}{C_{SS,mat} \times Cl_{S,mat}} \quad (\text{Equation 2.1}) \\
 &= \frac{F_{mat} \times (M/P) \times (V_{milk}/\tau)_{infant}}{Cl_{S,mat}}
 \end{aligned}$$

The RID has limitations as an exposure index. The RID fails to describe the systemic exposure of the nursing neonate to the drug, as neonatal pharmacokinetic processes including oral absorption and clearance must also be considered. A better measure of exposure risk is use of an index that considers both the bioavailable dose received by the nursing neonate and the ability of the neonate to eliminate that dose, i.e. the EI_{conc} . The EI_{conc} is an exposure index that relates neonatal plasma concentrations to maternal plasma concentrations. This exposure index assumes no qualitative differences in the plasma concentration versus response relationship between the neonate and the mother. Hence, equivalent concentrations at pharmacological levels suggest the neonate also experiences a similar pharmacological response (8). The EI_{conc} considers the ability of a neonate to eliminate the exposure dose.

$$EI_{Conc} = \frac{\bar{C}_{serum}^{infant}}{\bar{C}_{serum}^{maternal}} = \frac{F_{infant}}{Cl_{systemic}^{infant}} \left(\frac{M}{P} \right) \left(\frac{V_{milk}}{\tau} \right) \quad (\text{Equation 2.2})$$

Assessment of neonatal exposure risk is a difficult task due to changes in mammary gland function and the composition of breast milk during lactation period (35, 158) as well as the changes in the neonatal pharmacokinetics. The actual dose present in the breast milk can change with stage of lactation, although this factor

usually is not considered in assessments of M/P ratios. For example, fat and protein, two major components of breast milk that can influence milk levels of certain drugs, change with stage of lactation. Indeed, higher levels of several drugs including tricyclic antidepressants have been found in the hind milk (later milk) compared to the fore milk (initial milk) due to the higher level of fat content in the hind milk (159). In addition, transporters expressed in the lactating mammary epithelium experience change in both expression and activity with stage of lactation (97-98), which can lead to different drug concentrations in the breast milk. Changes in milk drug concentrations alter the M/S ratio and thereby change the exposure dose to the nursing neonate (158, 160). Therefore, lactation stage should be an important consideration in the risk assessment of drug exposure via breast milk.

Additionally, neonatal development is marked by dramatic changes in body composition, organ function and underlying physiological and biochemical processes that are important determinants of drug pharmacokinetics (158, 160). These changes have the potential to alter the extent of drug absorption (F_{neonate}) and/or eliminate ($Cl_{\text{infant,systemic}}$) drugs from their body, which can affect the exposure risk. Thus, postnatal ages should also be considered when drug exposure risk is assessed. Risk assessment is also further complicated by the lack of data on long-term adverse outcomes in neonates breastfed by mothers using medications (29).

2.5.2 Mechanisms of Drug Transport into Milk

The extent of drug transfer into breast milk depends on various factors including the physiochemical and pharmacokinetic characteristics of the drug, mechanism of its transport across the mammary epithelium, and the volume and composition of breast milk (161). Most drugs cross the blood-mammary-barrier via passive transport processes. However, some drugs cross this barrier via carrier-mediated transport processes (154). Depending upon their localization in the polarized epithelium (i.e. apical or basolateral expression) and their primary function (i.e. uptake vs. efflux), transporters (see section 2.3.3.1) may either accumulate drugs into breast milk or serve a protective role by preventing their access to the breast milk. *In vivo* studies have identified a role for transporters in the accumulation of several organic cations like cimetidine and ranitidine (29). Jonker et al. provided definitive evidence for the role of transporters as a critical determinant of the extent of drug accumulation into breast milk (162). In this study the authors demonstrated

the role of Breast Cancer Resistance Protein (BCRP, ABCG2) in the active secretion of the drugs, cimetidine and topotecan, as well as the dietary carcinogen PhIP, into milk. However, for many of the transporters known to be expressed in the lactating mammary gland (76, 83), their role in drug and nutrient transport requires elucidation. As to the other mechanisms of solute transport in the mammary gland (see section 2.3.3), these mechanisms play little to no role in drug transfer into the breast milk.

2.5.3 Neonatal Pharmacokinetics

The magnitude of the risk posed to the neonate following exposure to drug via the breast milk depends on the amount of drug in the milk and the pharmacokinetic and pharmacodynamic properties of the drug in the neonate (156, 161). Our understanding of qualitative (pharmacodynamic) differences in response to drugs during postnatal development is quite limited. In the absence of age-dependent differences in pharmacodynamics, exposure risk often correlates with drug concentration in the blood and total exposure is usually measured by the metrics, area-under-the-plasma-concentration-versus-time curve (AUC) or average steady state plasma concentrations ($C_{SS,ave}$). These exposure metrics depend upon the size of the bioavailable dose and the efficiency of the elimination mechanisms, which is given as systemic clearance (Cl_s) (Equation 2.3).

$$AUC_0^\infty = \frac{F \times \text{Dose}}{Cl_s} \quad \text{or} \quad C_{SS,ave} = \frac{F \times \text{Dose} / \tau}{Cl_s} \quad (\text{Equation 2.3})$$

The physiological and biochemical changes that occur during postnatal maturation results in dramatic developmental changes in drug pharmacokinetics. The most critical determinant of exposure risk is the extent of maturation of the drug elimination mechanisms (i.e. Cl_s). Most hepatic and renal elimination mechanisms, the principal pathways involved in drug elimination, undergo tremendous enhancements in function and efficiency with development (30-31). Consequently, the stage of maturation of these mechanisms in the nursing neonate will determine their capacity to eliminate an exposure dose. With limited maturation, even small exposure doses can lead to significant drug accumulation in the neonate to levels that are potentially toxic.

Since drug elimination mechanisms undergo constant maturation in the postnatal period, the timing of the exposure relative to the age of the neonate must factor into any consideration of exposure risk. For a given dose or amount of drug present in the breast milk, risk of adverse effects may be higher in the newborn than an older neonate because of the newborn's reduced capacity to eliminate the drug. Hence, neonatal pharmacokinetic processes can be a critical determinant of exposure risk.

2.5.4 Ontogeny of Nutrient Transporters

The ontogeny of nutrient transporters in neonates reflects their developmental need for nutrients required for growth and metabolism (163). Many nutrient transporters are expressed prenatally, and the ontogenic changes that occur with development include changes in turnover number, expression density, affinity constant, and/or expression of different isoforms in various organs (163). During breastfeeding, fatty acids provide the primary source of energy to the nursing neonate. Various transporters involved in the usage of fatty acid undergo change during the early postnatal period to guarantee the successful fuel switch from glucose to fatty acid. For example, the renal re-absorption of L-carnitine, an obligatory factor for fatty acid β -oxidation, is enhanced during the postnatal period (164). Cardiac fatty acid transporter expression increases significantly following parturition (37). Glucose transporter expression profiles change in various organs to meet the various glucose substrate demands of these organs (37, 165-166). In addition to the transporters responsible for energy substrate uptake and utilization, other nutrient transporters undergo ontogenic changes, although our understanding of such changes is quite limited.

Though developmental changes in nutrient transporters are mostly genetically programmed (167), the development of these transporters can still be reprogrammed by interactions with dietary factors (163). This suggests alterations in the breast milk composition of nutrients during early development may impact nutrient transporter ontogeny in the nursing neonate. For example, GLUT5 expression levels, which mediates fructose uptake from the gastrointestinal tract, increases during weaning under normal conditions (168). However, introduction of a high-fructose diet in early life further enhances its expression in the intestinal epithelium (168). Also, dietary fat

content has the capacity to affect intestinal membrane lipid composition and fluidity and thus affect activity of Na^+ -dependent D-glucose transport (169).

These nutrient transporters also play an important role in drug pharmacokinetics. Numerous commonly used drugs (such as antibiotics, antivirals, and non-steroidal anti-inflammatory agents) and physiologically important endogenous compounds (i.e. folate, choline, L-carnitine) are substrates of the same transporters. The ontogenic development of these transporters during postnatal life can be very important from both an endogenous function and pharmacokinetic point of view. For example, P-glycoprotein, an efflux transporter for digoxin, steadily increases in expression in kidney during the nursing period. These ontogenic changes are highly correlated with the enhancements in digoxin renal clearance with postnatal development (170).

2.5.5 Drug-Nutrient Interactions in the Nursing Neonate

All these drugs excreted into breast milk will be passed on to the nursing neonate. The majority of the available literature in the area of drug nutrient interactions in the infant population focus on the effect of food on the pharmacokinetic processes of the medications in the infancy and childhood (171). Little information is there regarding the effects of these drugs on the nutrients levels in the neonates. Drugs present in the breast milk can induce changes in nutrient supply to the nursing neonates by changing the taste of milk which can suppress the appetite, cause gastric irritation, and change neonatal GI functions (172). They can also interact with all the above nutrkinetic processes in the nursing neonates and therefore alter the nutrient level in the breastfed infants. Normally, drug induced nutritional deficiencies are slow to develop (173). However, insufficient supply of certain essential nutrients during critical developmental stages can lead to nutritional deficiency in the developing child. For example, exposure to the antiepileptic drug valproic acid (VPA) during adolescence can lead to folate deficiency though the mechanism underlying it is unknown yet (172).

2.5.6 Drug-Nutrient Transport Interactions: Cefepime-L-Carnitine Transport Interaction as Proof-of-Concept

Whether xenobiotics may influence breast milk composition by interference

with nutrient transport processes is not clearly understood. In addition to the interaction at the mammary gland, the presence of a xenobiotic in the breast milk may result in drug-nutrient transport interactions in the neonate. As proof-of-concept, my research project proposes the use of L-carnitine, a conditionally essential nutrient, and cefepime, a moderate L-carnitine transporter inhibitor, to determine whether a significant drug-nutrient transport interaction can occur *in vivo*.

2.5.6.1 L-Carnitine: Function and Biosynthesis

L-Carnitine, a water soluble quaternary amine that exists as a zwitterions under physiological condition, performs a crucial role in the β -oxidation of fatty acids. L-Carnitine controls the influx of long-chain fatty acids into mitochondria via the L-carnitine acyltransferase enzyme system (174-175). L-Carnitine can also affect the intermediary metabolism of fats, carbohydrates, and amino acids by acting as an intramitochondrial acyl-group acceptor, thus helping to regulate acyl-CoA/CoA ratios (176) and the mitochondrial respiratory chain (174), and can facilitate the oxidation of pyruvate due to stimulation of pyruvate dehydrogenase complex (174). L-carnitine, and its endogenous metabolite acetylcarnitine, may have a special function in the brain as they influence acetylcholine synthesis by controlling the acetyl moiety level (177).

Normally, L-carnitine is maintained at a steady level in the blood (178). This homeostasis is maintained by L-carnitine biosynthesis in the liver and brain, dietary L-carnitine, and renal reabsorption of filtered L-carnitine (Figure 2.2) (179). The mammalian L-carnitine pool is heavily dependent on exogenous intake: only about 25% of the L-carnitine source for the body comes from the liver and the kidney in man, whereas the remaining 75% comes from the diet (174). The precursors of L-carnitine are the two essential amino acids lysine and methionine (180). All enzymes in the pathway of L-carnitine biosynthesis are ubiquitous in mammalian tissues. However, γ -butyrobetaine hydroxylase, the enzyme that catalyzes the final step of the L-carnitine synthesis, is present only in a few tissues including liver (180).

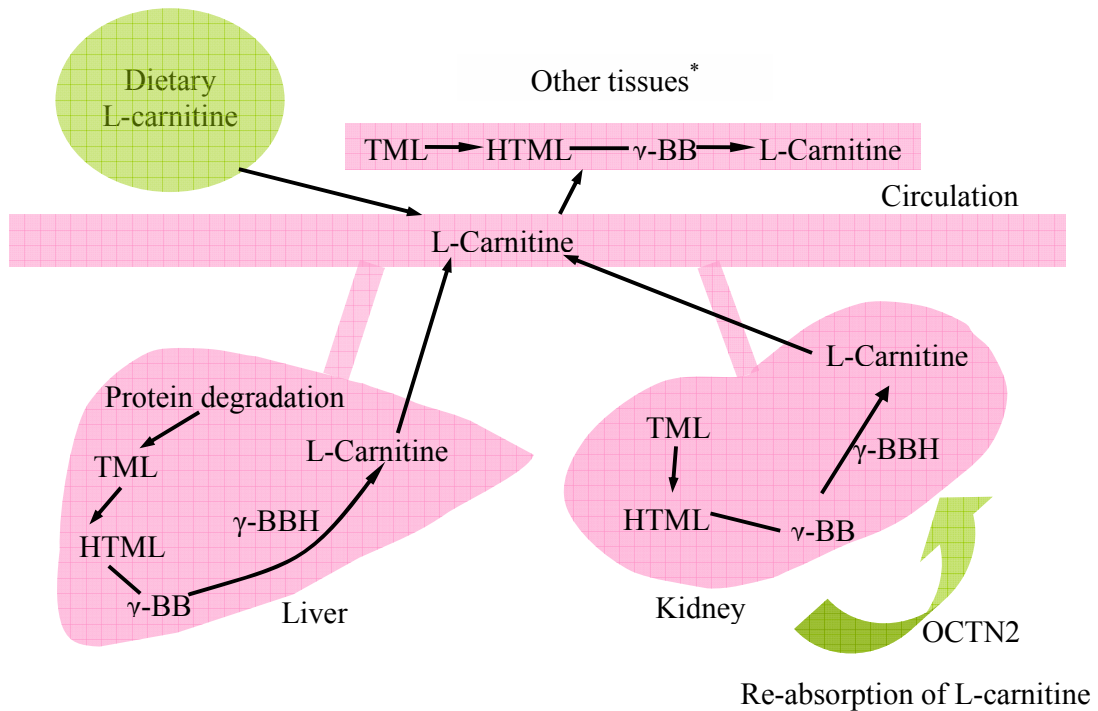


Figure 2.2. Schematic representation of L-carnitine homeostasis. TML: N⁶-trimethyl-lysine; HTML: 3-hydroxy- N⁶-trimethyl-lysine; γ-BB: γ- butyrobetaine; γ-BBH: γ- butyrobetaine hydroxylase; OCTN2, novel organic cation transporter 2. *L-Carnitine biosynthesis in other tissues is limited due to limited tissue distribution of γ-BBH. (Adapted from Vaz and Wanders (180))

2.5.6.2 L-Carnitine and the Neonate

In mammalian fetal tissues, metabolic energy is obtained mainly from carbohydrate metabolism (174). After birth, the newborn depends on a new source of nutrition (i.e. milk), which is high in fat and low in carbohydrate (37). The newborn must be able to respond to this change in energy substrates supply. Parturition signals the increase in expression of fatty acid transporters and enzymes of fatty acid metabolism in heart to allow fatty acids to become the major energy source for cardiac muscle (37). Improvements in fatty acid utilization are necessary to ensure normal growth and development (53). Fatty acid oxidation disorders are associated with the sudden infant death syndrome (181). Fatty acid oxidation also plays an important part in the thermogenic function of brown adipose tissue to maintain the thermal homeostasis (53). The L-carnitine dependent oxidation of long chain fatty acids is particularly important for the provision of acetyl units required for the

synthesis of complex lipids in the developing brain (182). The utilization of long chain fatty acids as a fuel source is dependent upon adequate concentrations of L-carnitine (183). The dramatic increase in fatty acid oxidation rates that occur in early postnatal heart development has been attributed to an increase in L-carnitine levels (184).

As in adults, the free L-carnitine concentration in the plasma of neonates probably represents an equilibrium between intestinal absorption, hepatic biosynthetic activity, uptake and release by tissues, and renal clearance (53). The plasma L-carnitine concentration of full term neonates increase during the first two weeks of life and reach adult levels by six months of age (185). At birth, the fetoplacental transfer of L-carnitine is interrupted. Before the onset of feeding the neonate heavily depends on fetal L-carnitine stores and on endogenous biosynthesis (174). However, the ability of neonates to biosynthesize L-carnitine is limited due to low levels of hepatic γ -butyrobetaine hydroxylase (γ -BBH) (186). The activity of γ -BBH increases with age and is only 10% -12% that of the normal adult levels at birth (53, 185). Thus, neonates are developmentally immature with regard to L-carnitine biosynthesis, and depend heavily on exogenous sources of L-carnitine. Merlegh et al. found that the L-carnitine content of pooled milk, despite its variability, was sufficient to maintain plasma levels in newborns without L-carnitine supplementation (187). However, any maternal drug affecting L-carnitine levels in the maternal system and thereby milk L-carnitine levels has the potential to cause inadequate or deficient L-carnitine levels in nursing neonates.

2.5.6.3 L-Carnitine Transporters

Uptake of L-carnitine into tissues may involve the OCTNs, $ATB^{0,+}$ and CT2 transporter systems. OCTNs, although showing rather high similarity of about 70% or more in amino acid sequences, function in distinct manners (188). OCTN1 is a multispecific, bidirectional, sodium-independent organic cation transporter in a pH-dependent manner, which has low affinity for L-carnitine transport in species including human and rat (189-191). It is expressed abundantly in rat intestine, liver and kidney (191). OCTN2 appears to be the major Na^{+} -dependent L-carnitine transporter common to mice, rat and human (192). The unique aspect of OCTN2 is its dual mode of transport: it transports L-carnitine via a Na^{+} -coupled process whereas transport organic cations via Na^{+} -independent (179, 193-194). OCTN2 is

widely expressed in human tissues such as heart, skeletal muscle, kidney, intestine, and placenta and brain (194-195). A mutation in OCTN2 gene has been considered as the causative factor for secondary L-carnitine deficiency (SCD). Compared to the wide distribution of OCTN1 and OCTN2, OCTN3 is unique in its limited tissue distribution which is by predominant expression in testis and Na⁺ independent L-carnitine transport (192, 196-197). Duran et al. suggested that OCTN3 could mediate the passive, Na⁺ and pH-independent L-carnitine transport activity (198). OCTN3 have a higher specificity for L-carnitine transport than OCTN1 and OCTN2 (192). Other than the OCTNs, ATB^{0,+} is also low-affinity transporter with a wide distribution in the intestinal tract and lung, as well as mammary gland (199-200). In addition, L-carnitine transporter 2 (CT2) is another novel L-carnitine transporter found recently. CT2 expresses specially in human testis with a reasonably high affinity toward L-carnitine (201). The localization of these transporters in tissues and at epithelial barriers is critical in understanding their endogenous role. For instance, OCTN2 is present on the apical membrane of rat intestinal epithelium, which suggests that OCTN2 is responsible for transporting L-carnitine into enterocytes (198). OCTN3 is expressed in peroxisomal membranes where it plays a critical role in peroxisomal β -oxidation pathways (202).

L-Carnitine concentrations in breast milk are higher than maternal plasma levels in the first two months after delivery (186). High M/P ratios usually suggest the active transport of that compound into breast milk. The L-carnitine transporters likely function in concert at the mammary epithelial barrier to produce a net accumulation of L-carnitine in breast milk during early lactation. In addition, the L-carnitine transporters also transfer other cation compounds such as therapeutic agents, drugs of abuse, and other xenobiotics that might be used by the mother during lactation. Thus, a thorough understanding of the L-carnitine transport system in the mammary gland and neonatal intestine would facilitate the evaluation of the pharmacological effects and toxicological potential of these compounds. These compounds have the potential to alter the L-carnitine tissue profile in the neonate, which, in turn, may have long-term effect on neonatal development.

2.5.6.4 L-Carnitine Deficiency

L-carnitine deficiency was first described as a new syndrome in humans in

1973 (203). Until now, two types of L-carnitine deficiency have been observed: primary and secondary L-carnitine deficiencies. These are commonly classified by the etiopathogenesis and organ involvement (203). Primary L-carnitine deficiency is caused by disturbances in the L-carnitine system itself and leads to impaired fatty acid oxidation (203). Primary L-carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation, which can be associated with a novel mutation in SLC22A5, the gene encoding OCTN2 (55, 204-205). Patients with primary L-carnitine deficiency have increased renal excretion of L-carnitine (205), and patients experience recurrent hypoketotic hypoglycemia and/or dilated cardiomyopathy (206-207). Secondary L-carnitine deficiencies are associated with an impaired mitochondrial oxidation of acyl-coenzyme A intermediates, a limited supply of substrates for L-carnitine biosynthesis, or a dietary insufficiency (208). Secondary L-Carnitine deficiency is defined as free plasma L-carnitine concentrations less than 20 $\mu\text{mole/L}$ or 0.39 mg/dL (209-210).

Toxicity caused by L-carnitine deficiency results from the accumulation of fatty acids or decreased energy production (211). Impairment in mitochondrial fatty acid oxidation can lead to severe hypoglycemia and dilated cardiomyopathy (212). In addition, patients with inborn errors of long chain fatty acid oxidation present with a variety of severe clinical problems, such as cardiomyopathy, retinopathy, and peripheral neuropathy, presumably due to the accumulation of toxic long chain acyl CoA esters (213). Symptoms related to a defect in OCTN2 may include fatty liver, growth retardation, hypoglycemia and hyperammonemia (178, 204, 214). Many individuals with L-carnitine deficiency are generally normal at birth and may appear healthy for several years until overt clinical signs appear (215). Early identification of L-carnitine deficiency can be lifesaving because L-carnitine supplementation during early stage can reverse certain pathological changes associated with cardiac, neural, and hepatic systems (215).

2.5.6.5 L-Carnitine and Cefepime Transport Interaction

Cefepime is a semi-synthetic cephalosporin used for parenteral human therapy (216). It is a powerful β -lactam antibiotic with excellent activity against a wide range of clinically important pathogens, particularly those strains resistant to other cephalosporins (217). Therapeutically, certain β -lactam antibiotics can induce a secondary L-carnitine deficiency due to inhibition of renal L-carnitine reabsorption

by OCTN2, thereby promoting the loss of L-carnitine in the urinary filtrate (218-219). In *in vitro* studies, cefepime competitively inhibits L-carnitine uptake via OCTN2 (219). Given these data, administration of cefepime to a lactating mother could result in the following significant drug-nutrient interactions: 1) Compete with L-carnitine for intestinal absorption and decrease L-carnitine bioavailability; 2) Compete with L-carnitine for renal tubular reabsorption and increase urinary loss of L-carnitine; 3) Inhibit L-carnitine transfer in the lactating mammary gland. The first two interactions have the potential to decrease maternal serum levels of L-carnitine and thereby decrease L-carnitine delivered to the mammary gland and into the breast milk. The third type of interaction can directly decrease milk L-carnitine levels. These drug nutrient interactions can occur simultaneously in the maternal system and result in more pronounced reductions in L-carnitine concentrations in breast milk, which could further compromise the nursing neonate's L-carnitine status.

3 Perspectives

Breastfeeding is highly recommended by international organizations such as WHO and FAO and is promoted by various health care professionals. However, maternal disease and its treatment may raise concerns regarding the safety of breastfeeding, which, in turn, may affect the decision of mothers to breastfeed their child. My research is designed to identify disease/drug-nutrient transporter interactions at the lactating mammary gland and in the nursing neonate. Such interactions can affect the levels of specific nutrients and drugs in the breast milk and nutrient availability and disposition in the neonate. Both these interactions can alter a neonate's nutritional status and consequently influence neonatal development as well as long-term health issues. In a practical sense, my research can provide valuable information for medication use during breastfeeding and during neonatal development for both pharmaceutical companies and various health organizations. Specifically, pharmaceutical companies can use this information to inform mothers about necessary precautions when using medications during breastfeeding. For example, my research may identify a need for dietary modification or supplementation when taking medications while breastfeeding to minimize the risk of nutrient deficiency in the breast milk. Moreover, my research may provide information regarding potential risks associated with maternal disease that alter mammary gland function and milk composition. This may then lead to better

nutritional supplementation for mothers with certain diseases, such as inflammation, who are still encouraged to breastfeed.

4 Hypothesis

Nutrient transporters expressed in mammary gland epithelium change during the lactation period to meet the nutritional requirements of the nursing neonate. Drugs may interfere with transporter function to cause changes in nutrient levels of breast milk. In addition, early life exposure to drugs may cause interactions that alter the nutrient homeostasis in the neonate and thereby interfere with normal biochemical and physiological development. Maternal health status may also affect mammary gland function and thereby change the milk composition. The effect of drug exposure and inflammatory conditions on nutrient transport in the lactating mother-neonate dyad is the focus of my research and I have the following hypotheses:

Hypothesis I: Nutrient transporter expression in the mammary gland epithelium changes with lactation period.

Hypothesis II: Drugs interfere with transporter function in the lactating mammary gland to cause changes in nutrient levels of breast milk.

Hypothesis III: Drug exposure during neonatal development modifies metabolic and biochemical pathways involving specific nutrients.

Hypothesis IV: Inflammatory stimuli alter mammary gland energy substrate transporter expression.

5 Objectives

Conditionally essential nutrients are critical for neonatal development. Co-administrated drugs may functionally interact with the transporters responsible for the transport of such nutrients across the lactating mammary epithelium and for absorption and disposition within the developing neonate. Such interactions may account for systemic and/or secondary deficiency in neonates. Therefore, it is important to clarify the transporters involved in the transfer of such nutrients into milk and uptake into and disposition within the neonate at different stages of lactation/neonatal development. By evaluating the potential interaction at various stages of lactation, we can gain further insight into when the interaction may be most

detrimental to the health of the nursing neonate. Furthermore, this information will also help us to understand the physiological roles of these nutrients in the neonatal development.

In my study, L-carnitine is used as an example of a critical nutrient in breast milk. Milk L-carnitine levels decrease as lactation stage advances. This may be associated with lactation stage dependent changes in L-carnitine transporter expression in the lactating mammary gland. Therefore, mRNA expression levels of various L-carnitine transporters in the lactating mammary gland will be analyzed at different lactation stages. In addition, the exact localization of these transporters in the polarized epithelium of the mammary gland is fundamental to our understanding of their physiological function in the mammary gland. Thus, the localization of selected L-carnitine transporters in the lactating mammary gland will also be identified (Objective 1).

L-Carnitine transporters also transport a variety of xenobiotics. Hence, xenobiotics can either inhibit nutrient (L-carnitine) transport or be transported into the breast milk. Therefore, an inhibitor of L-carnitine transport will be administered to lactating rat mothers at different lactation stages to determine whether an interaction changes L-carnitine milk levels and whether lactation stage affects the magnitude of this interaction (Objective 2).

Drugs present in breast milk will eventually be delivered to the nursing neonate. Compared to the adult, neonates undergo significant development and maturation. These changes are known to alter drug pharmacokinetics in the neonates compared to the adults, which may predispose the neonate to adverse effects. Thus, it becomes important to evaluate the pharmacokinetics of the chosen L-carnitine transport inhibitor in the neonate at different postnatal time points (Objective 3). With inefficient clearance processes even subtle amounts of xenobiotics in the milk may pose a long term health risk to the developing neonate, particularly when the exposure occurs during a “critical window” of development. As such, my aim is to identify potential biomarkers of toxicity to the L-carnitine inhibitor cefepime at different postnatal ages and for different duration of exposures (Objective 4).

In addition to maternal medication, maternal diseases are also a concern during breastfeeding. For example, inflammatory states such as mastitis are common during breastfeeding. Inflammation may create an unfavorable change in nutrient

composition of the breast milk and negatively affect lactation. In acknowledgment of this I also intend to estimate the potential effects of inflammation on the function of mammary gland energy substrate transporters using an *in vitro* human mammary gland epithelial cell culture system (Objective 5). Since the mammary gland epithelial functions during lactation are different compared to non-lactation stages, I will examine the effects of inflammation on energy substrate transporter expression *in vivo* using lactating rats at different lactation stages (Objective 6).

In summary, my proposed objectives are:

1. To characterize localization and expression levels of L-carnitine transporters in rat mammary gland at different lactation stages.
2. To measure L-carnitine levels in rat milk and serum in presence and absence of cefepime at different lactation stages.
3. To study cefepime pharmacokinetics following acute cefepime administration at different developmental stages in rats.
4. To measure biomarkers of L-carnitine homeostasis following chronic cefepime exposures at different developmental stages to identify potential programming effects with postnatal drug administration.
5. To measure energy substrate transporter mRNA expression levels in MCF12A when challenged by LPS or LTA.
6. To measure energy substrate transporter mRNA expression levels in mammary gland of lactating rats treated with LPS at different stages.

6 Acute Administration of Cefepime Lowered L-Carnitine Concentrations in Early Lactation Stage Rat Milk

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<http://jn.nutrition.org/cgi/content/full/138/7/1317/DC1>



Acute Administration of Cefepime Lowers L-Carnitine Concentrations in Early Lactation Stage Rat Milk¹⁻³

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Abstract

Our study investigated the potential for important *in vivo* drug-nutrient transport interactions at the lactating mammary gland using the L-carnitine transporter substrates, cefepime and L-carnitine, as proof-of-concept. On d 4 ($n = 6$ /treatment) and d 10 ($n = 6$ /treatment) of lactation, rats were administered cefepime (250 mg/h) or saline by continuous i.v. infusion (4 h). Serum and milk L-carnitine and cefepime concentrations were quantified by HPLC-UV. In whole mammary gland, organic cation/carnitine transporter (OCTN1, OCTN2, OCTN3, amino acid transporter B⁰+/ATB⁰+), and L-carnitine transporter 2 expression were determined by quantitative RT-PCR and by western blot and immunohistochemistry when possible. Cefepime caused a 56% decrease in milk L-carnitine concentrations on lactation d 4 ($P = 0.0048$) but did not affect milk L-carnitine at lactation d 10 or serum L-carnitine concentrations at either time. The mean L-carnitine and cefepime milk:serum ratios (M/S) decreased from 9.1 ± 0.4 to 4.9 ± 0.6 ($P < 0.0001$) and 0.89 ± 0.3 to 0.12 ± 0.02 ($P = 0.0473$), respectively, between d 4 and d 10 of lactation. In both groups, OCTN2 ($P < 0.0001$), OCTN3 ($P = 0.0039$), and ATB⁰+ ($P = 0.004$) mRNA expression and OCTN2 protein ($P < 0.0001$) were higher in mammary glands at d 4 of lactation compared with d 10. Immunohistochemistry revealed OCTN1 and OCTN2 localization in the mammary alveolar epithelium and OCTN3 expression in the interstitial space and blood vessel endothelium. In conclusion, cefepime significantly decreased milk L-carnitine concentrations only at d 4 of lactation. Relative to d 10, enhanced expression of OCTN2 and ATB⁰+ in mammary glands at d 4 of lactation and higher M/S (L-carnitine and cefepime) suggests cefepime competes with L-carnitine for L-carnitine transporters expressed in the lactating mammary gland to adversely affect L-carnitine milk concentrations and these effects depend upon lactation stage. J. Nutr. 138: 1317–1322, 2008.

Introduction

National and international programs advocate exclusive breast-feeding for the first 6 mo of life due to the considerable socio-economic and health benefits associated with breast-feeding. However, most mothers take 1 or more medications and supplements in the postpartum period and this has raised concerns about infant safety due to their transfer through the breast milk during lactation (1). Yet the recommendations on drug/supplement use during breast-feeding (2,3) are largely guided by experience of use, observational studies, epidemiological studies, case reports, and theoretical concerns. To provide additional data to improve the recommendations, most investigations in this area today aim to identify the extent and mechanism of transfer of such substances into milk and the subsequent relative infant dose following exposure via the breast milk (1,3,4). The relative infant

dose provides critical information regarding the possibility of adverse outcomes following infant exposure to compounds present in the breast milk (5). Although these efforts represent important achievements in the area, few studies have explored the potential negative outcomes of maternal medication use on mammary gland function (i.e. drug-nutrient interactions) and subsequent consequences on milk composition.

Drugs may disrupt milk volume and composition by altering the hormonal milieu necessary to support lactation, blood flow to the mammary gland, the amount of functional mammary tissue, or through direct or indirect interference with nutrient secretion by the mammary epithelium (6). In regards to the latter mechanism, recent studies have identified the expression of numerous solute carrier and ATP-binding cassette transporter families in the mammary gland during lactation (7,8). Their substrate profiles often include both nutrient and nonnutrient elements and various drugs (4). Consequently, the breast milk concentrations of many nutrients and some drugs are likely dependent upon the normal functioning of these transporters. Furthermore, a requirement for the same transport system makes possible the potential for reversible interactions between a drug and a nutrient. At other blood-epithelial barriers, drugs

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³ Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.

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and nutrients competing for the same transporter have resulted in reduced nutrient transport (9–11), which have sometimes necessitated a nutritional intervention (9,10). However, drug-nutrient transport interactions at the lactating mammary gland and the consequences of these interactions have received very limited attention.

The conditionally essential nutrient, L-carnitine, has an important role in mitochondrial utilization of long chain fatty acids for energy production. This micronutrient has particular importance in the developing cardiovascular and neurological systems of the nursing neonate of all mammalian species (12). L-Carnitine biosynthesis is developmentally immature in the newborn and neonates depend critically on breast milk sources (13). As a cation at physiological pH, intracellular availability of L-carnitine and its transfer across blood-epithelial barriers requires specific transport systems. Several transporters mediate L-carnitine transfer across membranes and these include the organic cation/carnitine transporters (OCTN)¹, OCTN2, and OCTN3, amino acid transporter system B^{0,+} (ATB^{0,+}), and the L-carnitine transporter 2 (CT2) (14–17). Substrates of these transporters also include the cationic drugs such as verapamil, pyrilamine, valproate, and the β -lactam antibiotics, cephaloridine, cefoselis, cefepime, and cefuprenam (18–20). In vitro studies in cell culture confirm a significant interaction between such compounds and L-carnitine for transport across cellular membranes (18–20).

As a proof-of-concept, the purpose of our study was to show an important in vivo drug-nutrient transport interaction at the lactating mammary gland between L-carnitine and a known inhibitor of L-carnitine transport, the β -lactam antibiotic, cefepime. We measured both milk and serum L-carnitine to identify whether an interaction would be specific to the mammary gland or whether it had systemic effects on the lactating rat. We also performed our studies at early and mid-lactation stages to determine the significance of the interaction at different lactation stages. Finally, we measured mRNA and protein expression of L-carnitine transporters in the rat mammary gland and compared the expression with the magnitude of the in vivo L-carnitine-cefepime interaction. A thorough understanding of L-carnitine transporter expression in the lactating mammary gland is critically necessary to elucidate its role in the transport of L-carnitine into milk and the toxicological potential of drug-nutrient transport interactions during the breast-feeding period.

Materials and Methods

Animals, diet, and chemicals. Female Sprague-Dawley rats ordered at gestation d 17 (for assessment of the cefepime-L-carnitine interaction at lactation d 4) ($n = 6$ per treatment) and lactation d 3 (for assessment of the cefepime-L-carnitine interaction at lactation d 10) ($n = 6$ per treatment) were obtained from Charles River Canada and were housed singly in a temperature- and humidity-controlled facility ($22 \pm 2^\circ\text{C}$) on a 12-h-light/dark cycle (0700–1900). All rats had free access to food and water throughout the study and were allowed a 7-d acclimatization period. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan.

Throughout the acclimatization period and during the study, rats consumed ad libitum a rat diet (Prolab RMH 3000, Purina) [% composition:

protein, 22.5; fat (ether extract), 4.5; fat (acid hydrolysate), 6.4; crude fiber, 4.0; ash, 6.1; calcium, 1.0; phosphorous, 0.75] that met the nutritional requirements for lactating Sprague-Dawley rats. No dietary manipulations were otherwise conducted and all dietary conditions were the same for the control and treated groups.

We purchased cefepime from the Royal University Hospital at the University of Saskatchewan (Saskatoon, SK). RNeasy Midi kits were obtained from Qiagen. We obtained all antibodies used in this study from Alpha Diagnostic International. The peroxidase substrate kit was purchased from Vector Laboratories. L-Carnitine and other chemicals not otherwise specified were obtained from Sigma-Aldrich.

Cefepime i.v. infusion study. A pilot study (total of 4 rats used) was conducted to optimize the time between pup removal from the dam and initiation and duration of cefepime infusion. This was necessary to ensure sufficient milk volume collection (particularly at 0 time) for analysis but to avoid dilution effects due to excessive milk accumulation in the mammary gland and intraday variation in L-carnitine concentrations. One day prior to the infusion study, the right jugular vein of each lactating dam was surgically cannulated (silastic tubing, 0.64-mm i.d. \times 1.19-mm o.d., Dow Corning) under isoflurane anesthesia. On lactation d 4 or 10, rat pups were removed from the dams 2 h before dosing. Cefepime (250 mg/h) or saline (control) was administered by continuous i.v. infusion (4 h) via the jugular catheter following an i.v. loading dose (50 mg). We drew blood samples just prior to initiation and termination of the infusion. Milk samples were collected by manual expression of the mammary gland under light isoflurane anesthesia. All samples were stored frozen (-20°C) until analysis. Following termination of the infusion, dams were humanely killed (by isoflurane overdose and thoracic cavity/cardiac severance) and mammary glands were rapidly excised. Representative sections of whole mammary gland were stored in RNAlater (Ambion), 10% paraformaldehyde, or flash frozen in liquid nitrogen and stored at -80°C until further processing.

L-Carnitine analysis. Milk and serum L-carnitine were quantified by HPLC-UV with precolumn derivatization according to Feng et al. (21) but modified to allow a 20- μL milk or serum sample (instead of 100 μL). The HPLC system was the same as described below for cefepime analysis. The standard curve range was 2.5–40 $\mu\text{mol/L}$ and the limit of detection was 0.16 $\mu\text{mol/L}$. Intra- and interassay accuracy and precision ranged from 6 to 14%.

Cefepime analysis. Milk and serum cefepime concentrations were measured with HPLC-UV. In screw-top glass culture tubes, 20 μL of rat plasma or milk was added with 180 μL acetonitrile:methanol (9:1) and mixed well. The mixtures were vortex-mixed (5 min) and centrifuged at $10,000 \times g$; 20 min. The supernatant was transferred to HPLC vials and 10 μL was injected onto the cyano column (HyperClone 5 μm , 250×4.6 mm, Phenomenex) with methanol:double distilled water (1:1) as the mobile phase delivered at 1 mL/min. The UV wavelength used for detection was 260 nm. The standards were prepared in male rat serum as described above. The standard curve ranged from 3.1 to 100 mg/L and the limit of detection was 0.20 mg/L. Intra- and interassay accuracy and precision ranged from 5 to 14%. Quality control samples at 3 different concentrations performed in duplicate were assessed as acceptance criteria for individual HPLC analyses.

Observed milk:serum ratios. We determined observed milk:serum ratios (M/S) of L-carnitine and cefepime from the ratio of the serum and milk concentrations (C) as follows:

$$\frac{M}{S} = \frac{C_{\text{Milk}}}{C_{\text{Serum}}} \quad (\text{Eq. 1})$$

The mean M/S for L-carnitine was calculated for each lactation day by using the serum and milk concentrations determined in both control and treated groups before the start of the saline (control) or cefepime (treated) infusion (i.e. the 0 h milk and serum concentrations such that $n = 12$ for L-carnitine M/S determination for each lactation day). For cefepime, the M/S was calculated in treated rats using the ratio of the milk and serum concentrations of cefepime determined at the end of the 4-h cefepime infusion ($n = 6$).

¹ Abbreviations used: ATB^{0,+}, amino acid transporter B^{0,+}; BSA, bovine serum albumin; CT2, L-carnitine transporter 2; M/S, milk:serum concentration ratio; OCTN, organic cation/carnitine transporter; QRT-PCR, quantitative RT-PCR.

Total mRNA isolation and quantitative RT-PCR analysis. Total mRNA was extracted from mammary gland stored in RNAlater solution using RNeasy Midi kits according to the manufacturer's instructions. RNA purity and quantity were determined spectrophotometrically by measurement at 260 nm and the OD₂₆₀/OD₂₈₀, respectively. Specific primers (Supplemental Table 1) for all OCTN, ATB^{0/+}, and CT2 were designed using Primer3 software (22). We conducted quantitative RT-PCR (QRT-PCR) analysis using a QuantiTect SYBR Green RT-PCR kit (Applied Biosystems) and an Applied Biosystems 7300 Real-Time PCR system. Real-time PCR assays were optimized to give PCR efficiency between 1.9 and 2.1 and a single melt-peak corresponding to the appropriate PCR product as verified by 2% agarose gel electrophoresis. Fold differences in mRNA expression were calculated using the standard curve method for relative quantitation with expression normalized to β -actin. Control tissues were obtained from male Sprague-Dawley rats at a similar age as the lactating dams.

Western blot analysis. Proteins were extracted from 300 mg of mammary gland tissue and reference tissues (kidney and testis from male Sprague-Dawley rats). Briefly, samples were homogenized in Radio-Immuno precipitation assay buffer (50 mmol/L Tris, 150 mmol/L NaCl, 10 mmol/L EDTA, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4) plus Protease Inhibitor Cocktail (Sigma-Aldrich) and 1 mmol/L phenylmethanesulfonylfluoride. The homogenates were incubated in ice for 1 h and centrifuged at 12,000 \times g; 20 min at 4°C. The supernatants were collected in new glass culture tubes. Protein concentrations were estimated using Bradford Reagent (Sigma-Aldrich). Forty micrograms of protein was separated on a sodium dodecyl sulfate gel containing 10% polyacrylamide (SDS-PAGE) by standard methods and transferred onto 0.2 μ m polyvinylidene difluoride membranes with a Bio-Rad Trans-Blot (Bio-Rad Laboratories) at 25 V for 80 min. Membranes were incubated with 3% bovine serum albumin (BSA) in 1 \times TBST (Tris-buffered saline with 0.1% Tween-20) for 1 h at room temperature. Primary antibody (rabbit anti-mouse OCTN IgG Affinity-pure for OCTN1, OCTN2, and OCTN3; Alpha Diagnostic International) was diluted as recommended by the manufacturer (1:1000) in 3% BSA in 1 \times TBST. Membranes were blotted in the primary antibody solution overnight at 4°C with constant shaking. The membrane was then washed in 1 \times TBST for 20 min 4 times at room temperature. Membranes were incubated with product labeled as (H+L)-peroxidase-conjugated affinity-pure goat anti-rabbit IgG (1:5000 in 3% BSA in 1 \times TBST; Alpha Diagnostic International) for 1 h at room temperature followed by washing in 3% BSA in 1 \times TBST for 1 h. After 4 washings with 1 \times TBST for 20 min at room temperature, the membrane was then transferred to the ECL Western Blotting Detection Reagents and Analysis system (Amersham Biosciences) for 1 min. The solution was removed and the membrane was then exposed to film (Kodak BioMax XAR) for 5 min. The density of each band was measured by using the ImageJ program (23). The specificity of each antibody was determined by overnight incubation of primary antibody with control/blocking peptide (Alpha Diagnostic International) before application to the membrane.

Immunohistochemistry. Kidneys and mammary glands from lactating dams and testis from male rats were fixed with 4% paraformaldehyde and paraffin-embedded sections (5 μ m) prepared using standard procedures. Sections were incubated with OCTN antibodies (1:100) (Alpha Diagnostic International) for 1 h and then (H+L)-peroxidase-conjugated affinity-pure goat anti-rabbit IgG (1:500) for 30 min. Immunoreactions were visualized by using VIP Substrate kit for peroxidase (Vector Laboratories) according to the manufacturer's instructions. We tested nonspecific binding by using premixed control peptide with the respective OCTN antibodies. For negative controls, only secondary antibody was applied to the slides.

Statistical analysis. All data are means \pm SEM. Two-way ANOVA (day \times treatment) was used to assess differences in milk and serum L-carnitine concentrations. When a main effect or the interaction was significant, a Tukey's studentized range test (honestly significant difference) was used for post hoc comparisons. Differences in L-carnitine M/S at d 4 and 10 of lactation were analyzed with an unpaired *t* test and

cefepime M/S was analyzed using an unpaired *t* test with Welch's correction. Differences in individual L-carnitine transporter expression between d 4 and 10 of lactation were analyzed with an unpaired *t* test. We used a paired *t* test to assess differences in the milk or serum concentrations of L-carnitine at the initiation and termination of the 4-h saline infusion (control rats) within a specific lactation stage. The significance level was $\alpha = 0.05$.

Results

In vivo cefepime-L-carnitine interaction study. In control rats, milk L-carnitine concentrations were \sim 100% higher ($P = 0.0083$) at lactation d 4 than at d 10 and serum L-carnitine concentrations did not differ between the 2 lactation stages (Table 1). Milk and serum L-carnitine milk concentrations in control rats did not change between the initiation and the termination of the 4-h infusion (data not shown). Cefepime caused a 56% decrease in L-carnitine milk concentrations at d 4 of lactation ($P = 0.0048$) but no change at d 10 (Table 1). At both d 4 and 10 of lactation cefepime administration did not alter serum L-carnitine concentrations (Table 1). In control rats, L-carnitine M/S at d 4 of lactation was 9.1 ± 0.4 , which was higher than the M/S of 4.9 ± 0.6 at d 10 of lactation ($P < 0.0001$). Cefepime M/S was also higher in treated rats at d 4 of lactation with an M/S of 0.89 ± 0.3 compared with 0.12 ± 0.02 at d 10 of lactation ($P = 0.0473$).

Expression of L-carnitine transporters. QRT-PCR analysis of OCTN1, OCTN2, OCTN3, ATB^{0/+}, and CT2 in whole mammary gland of control and treated rats at lactation d 4 and 10 revealed that OCTN1 and CT2 expression were similar at both lactation stages (Table 2). However, expression of OCTN2, OCTN3, and ATB^{0/+} in whole mammary gland was significantly higher at lactation d 4 than at d 10 ($P < 0.05$) (Table 2).

Western blot analysis of the OCTN transporters in control and treated rat whole mammary gland revealed no changes in OCTN1 and OCTN3 expression between lactation d 4 and 10 (Fig. 1). However, OCTN2 protein in whole mammary gland was \sim 100% higher at lactation d 4 compared with d 10 (Fig. 1).

Immunohistochemical analysis showed that OCTN1 was mainly localized on the blood vessel endothelium as well as the secretory alveolar apical membranes in the mammary gland (Fig. 2). However, slight staining for OCTN1 was also observed on the secretory alveolar basolateral membranes in the mammary gland. OCTN2 expression was observed in the lactating mammary gland, but we could not definitively determine the localization of OCTN2 on the apical or basolateral membranes of the secretory alveoli of the mammary gland (Fig. 2). OCTN3

TABLE 1 Serum and milk free L-carnitine concentrations in rats administered saline (control) or cefepime (treated) (250 mg/h with a 50-mg loading dose) i.v. for 4 h at d 4 or 10 of lactation¹

Lactation day	Serum		Milk	
	Control	Treated	Control	Treated
	$\mu\text{mol/L}$			
4	30.5 \pm 1.7	23.8 \pm 2.6	211 \pm 29	91.6 \pm 16*
10	27.4 \pm 3.1	24.1 \pm 2.2	111 \pm 9.4 [#]	107 \pm 10

¹ Values are means \pm SEM, $n = 6$. *Different from control, $P = 0.0048$; [#]different from d 4, $P = 0.0083$. For serum data, time, treatment, and their interaction were not significant.

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TABLE 2 mRNA expression of L-carnitine transporters (OCTN1, OCTN2, OCTN3, ATB^{0,+}, and CT2) in Sprague-Dawley rat whole mammary gland at d 4 and 10 of lactation¹

Lactation day	OCTN1	OCTN2	OCTN3	ATB ^{0,+}	CT2
4	7.0 ± 1.0	8.1 ± 0.9	4.3 ± 0.5	16 ± 3.5	0.21 ± 0.04
10	8.5 ± 2.2	3.4 ± 0.3*	2.5 ± 0.3*	1.2 ± 0.2*	0.30 ± 0.05

¹ Values are means ± SEM, n = 12 control and treated rats. *Different from d 4, *P* < 0.004.

was highly expressed in the interstitial space as well as in the blood vessel endothelium (Fig. 2).

Discussion

The risk of nutrient deficiencies in the infant following drug- or toxicant-induced changes in breast milk composition has received limited attention (24–26). Yet, breast milk concentrations of many solutes (i.e. nutrients, drugs) (27) depend upon the activity of a variety of solute carrier and ATP-binding cassette transporters expressed at the lactating mammary epithelium (7,28). Although adverse drug-nutrient transport interactions are known to occur at other epithelial barriers (i.e. gastrointestinal tract, kidney, liver, blood-brain barrier) (10,11), our unawareness of the potential for such interactions at the lactating mammary epithelial barrier represents a fundamental knowledge gap. Animal studies have substantiated the impact of unbalanced nutrition on epigenetic and metabolic programming of biochemical and physiological pathways of the infant, particularly during critical developmental windows (29–32). Any maternal factor (i.e. disease, diet, exposure to xenobiotics) (25,26,33,34) that adversely alters breast milk volume and/or composition could have serious consequences on infant development because of essential nutrient deficiencies in the mother's milk (31,35–37).

In this study, we focused on the conditionally essential nutrient, L-carnitine, because newborns depend almost exclusively on maternal milk for their L-carnitine requirements (38–40). Also, L-carnitine is actively transported into breast milk (28,41), resulting in high milk concentrations relative to maternal blood concentrations, particularly in the first few months of lactation (7,13,28,41–44).

In our study, L-carnitine and cefepime M/S were higher at d 4 of lactation, which is consistent with data reported in the literature (13,28,41–44). Furthermore, cefepime significantly decreased the milk concentration of L-carnitine at d 4, but not d 10, of lactation. The higher M/S and the ability of cefepime to affect L-carnitine milk concentrations at d 4 of lactation may relate to the observed changes in the expression levels of the major L-carnitine transporters in the mammary gland with stage

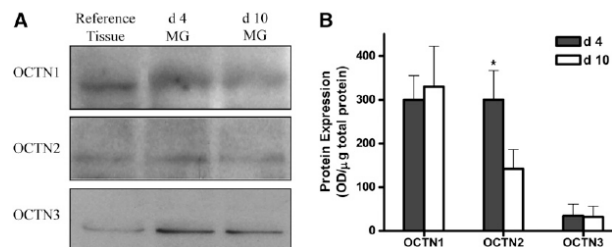
of lactation (28,41,44). Although OCTN1 and CT2 mRNA expression remained constant, OCTN2, OCTN3, and ATB^{0,+} expression in the mammary gland was greater at d 4 of lactation than at d 10. In human mammary gland epithelia cell cultures (MCF12A), hOCTN2 and ATB^{0,+} principally mediate the active uptake of L-carnitine into cells (44). Therefore, the diminished expression of OCTN2 and ATB^{0,+} in lactation d 10 mammary glands may explain the reductions in L-carnitine and cefepime M/S and the inability of cefepime to alter L-carnitine milk concentrations at d 10 of lactation. These conclusions are supported by the immunohistochemical localization of OCTN1 and OCTN2 in the rat mammary alveolar epithelium (Fig. 2), which is consistent with expression reported in human mammary gland (44).

Interestingly, we observed strong expression of OCTN3, a high affinity L-carnitine transporter with known limited tissue distribution (i.e. kidney, intestine, and testis) (45,16,46), in blood vessel endothelium and in the interstitium of the mammary gland. OCTN3 has expression in peroxisomes (46,47), where it participates in peroxisomal β -oxidation by transporting short-chain fatty acids out of the peroxisome (48). OCTN3's role in the rat mammary gland is not clear but may have importance in overall fatty acid oxidation and cellular energy production (49,50).

Although the short cefepime infusion (4 h) caused significant changes in milk L-carnitine concentrations at d 4 of lactation, maternal systemic (i.e. blood) concentrations remained unchanged. Inhibition of active renal reabsorption of L-carnitine causes excessive L-carnitine loss (32) and long-term treatment with L-carnitine transporter inhibitors (i.e. valproic acid) may lead to secondary L-carnitine deficiencies (51). Our data suggest the short-term exposure to cefepime did not cause a significant acute interaction with the renal tubular L-carnitine transporters. However, with chronic administration (the typical therapeutic situation), sustained cefepime-L-carnitine transport interactions at both the lactating mammary and renal tubular epithelia could result in more pronounced reductions in L-carnitine concentrations in breast milk, which would further compromise the nursing infant's L-carnitine status.

When placed within the context of infant exposure risk during breast-feeding, cefepime administration to the mother during early lactation may pose a greater risk to the nursing infant than cefepime use later in lactation. According to our data, cefepime would affect milk L-carnitine concentrations early in lactation to reduce L-carnitine availability to the nursing infant (nutritional deficiency). At the same time, higher milk concentrations of cefepime in early lactation would enhance cefepime exposures in the nursing infant (pharmacological toxicity). This exposure is further compounded by the immaturity of the infant's drug elimination mechanisms in early postnatal life (52). Inefficient elimination of cefepime in the developing infant can result in

FIGURE 1 (A) Western blot analysis of OCTN transporters isolated from reference tissue and mammary gland (MG) at lactation d 4 and 10 combining both control and cefepime-treated (250 mg/h for 4 h with a 50-mg loading dose) dams. (B) Western blot analysis of OCTN transporter expression (OD/ μ g total protein) in mammary gland at lactation d 4 and 10. Values are means ± SEM, n = 12. *Different from lactation d 10, *P* < 0.05.



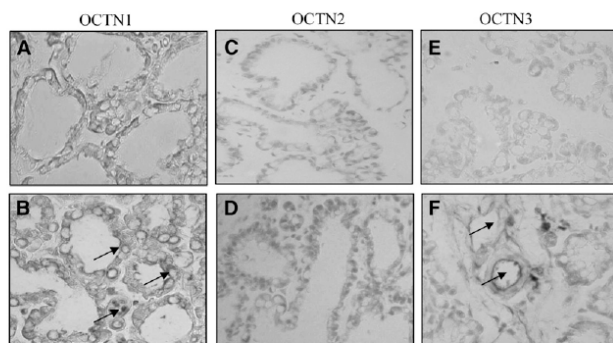


FIGURE 2 Localization of OCTN1, OCTN2, and OCTN3 proteins in the lactating mammary gland of a lactation d 4 control rat by immunohistochemistry. (A,C,E) OCTN antibody (dilution 1:100) preincubated with the appropriate OCTN control peptide (100 mg/L). (B,D,F) OCTN antibody (dilution 1:100); magnification 400 \times . OCTN1 (A,B) was mainly localized on the blood vessel endothelial cell membrane as well as the secretory alveoli apical membrane in the mammary gland (as shown by the arrow in B). However, slight staining for OCTN1 was also found in the secretory alveoli basolateral membranes. OCTN2 (C,D) was localized to the secretory alveolar membranes of the mammary gland. However, the tissue sections failed to distinctively identify whether OCTN2 had apical or basolateral expression. OCTN3 (E,F) was highly expressed in the interstitial space as well as on blood vessel endothelium (as indicated by the arrow in F).

cefepime accumulation to potentially toxic levels and interference with endogenous L-carnitine transport function in the nursing infant. Lower L-carnitine coupled with higher cefepime milk concentrations in early lactation may have a considerable influence on the nursing infant's L-carnitine status. Hence, lactation stage can be a critical factor when evaluating infant exposure risks after maternal medication use during breast-feeding.

How cefepime-mediated reductions in L-carnitine milk concentrations influence infant development remains unknown and warrants investigation. Such investigations are particularly relevant, because research draws an ever-compelling association between early life nutrition and the origin of diseases like cardiovascular disease, obesity, and type II diabetes (31,32,53). Through regulation of gene expression, dietary factors can alter disease initiation or progression. Even short-lived nutritional imbalances have lasting influences on health when such stimuli occur at critical developmental windows (54). Studies have shown that reduced L-carnitine availability can alter gene expression and activity of key proteins involved in long-chain fatty acid metabolism in rats (55). In human infants < 4 mo of age, lack of dietary L-carnitine affects lipid metabolism and FFA concentrations (56). However, dietary supplementation with L-carnitine corrects impairment in fatty acid oxidation in patients with L-carnitine deficiency due to defects in OCTN2 function (57). Interestingly, individuals with L-carnitine deficiency can remain asymptomatic for several years before overt clinical signs occur (58). Yet, early identification of L-carnitine deficiency may be critical, because infantile-onset cardiomyopathy, as a result of L-carnitine deficiency due to an OCTN2 transporter defect, is preventable by dietary L-carnitine supplementation (58). Because the postnatal period represents a vulnerable stage of development, any factor affecting milk concentrations of L-carnitine and subsequent L-carnitine status of the infant could have important health consequences on the developing individual (31,36,37).

In summary, our study provided proof-of-concept for the ability of a drug to adversely affect the micronutrient composition of breast milk through possible competitive interactions with nutrient transporters in the lactating mammary gland. Cefepime's ability to reduce milk concentrations of L-carnitine at one stage of lactation (i.e. d 4) but not at another (i.e. d 10) emphasizes the importance of examining competitive drug-nutrient transporter interactions at different lactation stages. Future studies should investigate other relevant drug-nutrient transport interactions at the lactating mammary gland and the health consequences of such interactions on the breast-feeding

mother-infant dyad. Eventually, such research may identify occasions when nutritional interventions are required to overcome nutrient deficiencies in the breast-feeding mother-infant dyad imposed by an interacting maternal medication.

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7 Lactation-Stage Influences Drug Milk-to-Serum Values and Neonatal Exposure Risk

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7.1 Abstract

Drug milk-to-serum concentration ratio (M/S) is a key determinant of Exposure Indices used to assess the risk associated with neonatal exposure to drugs present in breast milk when nursing mothers take medications. The primary purpose of our study was to assess experimentally the influence of lactation stage dependent differences in M/S for an actively transported drug on the calculation of two Exposure Indices, EI_{Dose} and EI_{Conc} . Cefepime was given to rat pups by intravenous (20 mg/Kg) or oral (40 mg/Kg) bolus administration at postnatal age day 4 and 10. Blood samples were collected via cardiac puncture at 0, 10, 20, 30, 45, 60, 120, 240, 360, and 480 min post-administration (1 pup/time point, $n=6$ /time). Cefepime concentrations were measured by HPLC-UV. Following IV administration, the elimination rate constant, half-life, and systemic clearance were significantly higher in 10 day-old versus 4 day-old rat pups. Cefepime M/S was higher at day 4 lactation versus day 10 (data from Ling and Alcorn 2008). The EI_{Conc} was quantitatively higher than the EI_{Dose} at both lactation stages. The ~7-fold decrease in cefepime M/S values at lactation day 10 resulted in ~7-fold reduction in the calculated EI_{Dose} (considering age-dependent changes in body weight and the volume of milk consumed per day) and ~13-fold reduction in the EI_{Conc} (considering age-dependent increases in systemic clearance and the volume of milk consumed per day). Our study experimentally confirms the need to evaluate M/S at different lactation stages for actively transported drugs to avoid over- or underestimation of the neonatal exposure risk.

7.2 Introduction

All chemicals have the potential to cross the lactating mammary epithelial barrier into milk (150-152). Any chemical (i.e. drugs, environmental toxicants) transferred into breast milk may pose a health risk to a nursing neonate. To assess this risk, the literature proposes several Exposure Indices that variably consider the key determinants affecting neonatal exposure to chemicals in breast milk (8, 150, 220). Both maternal factors (i.e. the bioavailable (F) maternal dose, maternal elimination capacity or systemic clearance (Cl_s), and the milk-to-serum concentration ratio (M/S)) and neonatal factors (i.e. the volume of milk consumed during nursing (V_{milk}/τ), the bioavailable neonatal dose, and neonatal systemic clearance) determine neonatal exposure risks during breastfeeding. The most commonly referred to Exposure Index, $EI_{(Dose)}$, bases risk assessment on the dose a neonate receives from the mother during nursing. To provide some relative measure of risk the $EI_{(Dose)}$ expresses neonatal dose as a percentage of the maternal dose as shown in Equation 7.1 (8):

$$\begin{aligned} EI_{(Dose)} &= \frac{\text{Absolute Neonatal Dose (mg/Kg/day)}}{\text{Maternal Dose (mg/Kg/day)}} \times 100\% \\ &= \frac{F_{Mat} \times C_{SS,Mat} \times (M/S) \times (V_{milk}/\tau)_{Neo}}{Cl_{S,Mat}} \end{aligned} \quad (\text{Equation 7.1})$$

where F_{Mat} is the maternal bioavailability, $C_{SS,Mat}$ is the steady state maternal serum concentration, M/S is the milk concentration-to-serum concentration ratio, $(V_{milk}/\tau)_{Neo}$ is the volume of milk consumed by the neonate per day, and $Cl_{S,Mat}$ is the maternal systemic clearance. For pharmaceutical agents, current guidelines suggest that drugs are safe to use during breastfeeding if the $EI_{(Dose)}$ is less than 10% (221). Since this risk assessment tool identifies the % of the maternal dose made available to the neonate it largely ignores the important contribution of neonatal pharmacokinetic processes as key determinants of neonatal exposure risk to chemicals present in the breast milk (8). However, this Exposure Index is more easily applied since published literature on maternal pharmacokinetics, milk-to-serum ratios and milk consumption rates may be available for a variety of drugs and a few environmental toxicants (222)..

A more comprehensive Exposure Index is the EI_{Conc} , (223), where risk is based

on the systemic exposure of the neonate to the chemical. To provide a relative measure of risk, the EI_{Conc} expresses neonatal steady state serum concentration as some percentage of the maternal steady state serum concentration as shown in Equation 7.2 (8):

$$EI_{(Conc)} = \frac{(\text{Steady State Serum Concentration})_{Neo}}{(\text{Steady State Serum Concentration})_{Mat}} \times 100\% \quad (\text{Equation 7.2})$$

$$= \frac{F_{Neo} \times (M/S) \times (V_{milk} / \tau)_{Neo}}{Cl_{S,Neo}}$$

where F_{Neo} is the neonatal bioavailability and $Cl_{S,Neo}$ is the neonatal systemic clearance. Systemic exposure often is a better indicator of toxicological outcomes than the size of the exposure dose as not only size of dose but the pharmacokinetic processes acting on that dose determine the concentration of a chemical at the toxicological site of action. As a risk assessment tool, the EI_{Conc} likely provides a better prediction of risk to the nursing neonate. However, this Exposure Index demands knowledge of neonatal pharmacokinetics, information that is often lacking in paediatric populations. This is further complicated by the dynamic changes in body composition, organ function and physiological and biochemical processes that occur with maturation and which cause age-dependent changes in pharmacokinetic characteristics of chemicals in the body (160).

McNamara and Abassi (8) recently reviewed the theoretical use of these exposure indices and how the different determinants of exposure risk influence the values of $EI_{(Dose)}$ and EI_{Conc} . This review highlighted the importance of including neonatal pharmacokinetics to more accurately reflect the risk as the EI_{Conc} bases the risk assessment on systemic concentrations and not simply on the ingested dose (8). What was of further interest was the importance of the M/S on the value of these exposure indices (see Equations 7.1 and 7.2) where the higher the M/S value (i.e. greater the compound distribution into milk), the higher the exposure. The M/S has been evaluated for a number of drugs. However, these are often determined without consideration of stage of lactation. Although the literature acknowledges the M/S variability between colostrum and mature milk (224-226), foremilk and hindmilk (227-229) and within a dosing interval (227), limited discussion exists about the stage of lactation (i.e. early,

mid, late) and its impact on the M/S value (35).

Many drugs distribute into milk via passive processes. In such cases the physico-chemical properties of the compound, fat partitioning, and plasma protein binding determines the milk levels of a compound (35) and often M/S values do not exceed unity. However, some drugs are substrates for transporters expressed in the lactating mammary epithelium (83, 95, 162). Actively transported drugs can achieve M/S values well exceeding 2 (158, 230). Recently our laboratory has shown clear evidence of changes in M/S values of an actively transported drug substrate with lactation stage due to lactation-stage dependent changes in the expression of transporters in the lactating mammary epithelium (1). Reports of transporter expression in the lactating mammary gland also clearly indicate significant changes in transporter expression levels with lactation stage (75, 97-98). Herein we provide experimental support for the need to consider lactation stage in the M/S determination of actively transported compounds and in the assessment of neonatal exposure risk with $EI_{(Dose)}$ and EI_{Conc} using cefepime, a β -lactam antibiotic substrate of organic cation/carnitine (Octn) transporters, and the lactating rat dam-pup dyad.

7.3 Materials and Methods

Animals and Chemicals. Female Sprague-Dawley rats ordered at gestation day 17 (for pups at postnatal day 4) (n=12) and lactation day 3 (for pups at postnatal day 10) (n=12) were obtained from Charles River Canada (St. Constant, PQ) and were housed singly in a temperature and humidity controlled facility ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) on a 12-hour light: dark cycle (0700 h – 1900 h). All rats had free access to food (Prolab® RMH 3000, Purina, Inc., Richmond, IN) and water throughout the study and were allowed a 7-day acclimatization period. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethics Board of the University of Saskatchewan. Cefepime was purchased as a sterile crystalline powder equivalent to 2 g of cefepime per vial from the Royal University Hospital at the University of Saskatchewan (Saskatoon, SK). HPLC solvents were HPLC grade and all other chemicals were highest analytical grade possible. A MilliQ Synthesis (Millipore, Bedford, MA) water purification system

provided purified deionized water.

Cefepime Pharmacokinetics in Rat Pups. Rat pups at postnatal age day 4 and day 10 were removed from their dams at least two hours prior to cefepime administration. These dams were used to assess steady state milk levels of cefepime at lactation day 4 and day 10 as reported in Ling et al. (1). Cefepime was reconstituted with sterile saline just prior to administration and diluted according to pup body weight. For intravenous injection, 20 mg/kg body weight in 50 μ L was administered via intracardiac puncture under isoflurane anaesthesia using a 27-gauge needle. For oral administration, 40 mg/kg body weight in 100 μ L was administered via a 24-gauge 1 inch gavage needle. Blood samples were collected from pup heart via cardiac puncture (under isoflurane anaesthesia) at 0, 10, 20, 30, 45, 60, 120, 240, 360, 480 min after administration and the pups were immediately euthanized. Each rat pup in a litter was assigned to a single time point following drug administration (i.e. destructive sampling scheme). Each litter was considered as n=1 and a total of 6 litters were used for each route of administration. Blood samples were allowed to clot for 30 min at room temperature and serum was collected following centrifugation at $1000 \times g$ for 10 minutes. Serum was stored in eppendorf microcentrifuge tubes at -20°C until analysis.

Cefepime Analysis. Milk and serum cefepime concentrations were measured by HPLC-UV. In screw-top glass culture tubes, 20 μ L of rat plasma or milk was added with 180 μ L acetonitrile: methanol (9:1), vortex-mixed for 5 min and centrifuged at $10,000 \times g$ for 20 min. The supernatant was transferred to HPLC vials and 10 μ L was injected onto a CN (cyano) column (HyperClone 5 μ m, 250×4.6 mm, Phenomenex, Torrance, CA) with methanol: purified deionized water (1:1) as mobile phase delivered at 1 mL/min. UV detection wavelength was set at 260 nm. The standards were prepared in male rat serum as above. The standard curve was linear ($r^2 > 0.99$) in the range of 3.1 mg/L-100 mg/L. Intra- and interassay accuracy and precision ranged from 5%-14%. Quality control samples at three different concentrations performed in duplicate were assessed as acceptance criteria for individual HPLC analyses.

Pharmacokinetic Analysis. The pharmacokinetic parameters of cefepime were characterized using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Plots of average serum cefepime concentration following oral or IV administration to Sprague

Dawley rat pups at postnatal day 4 and postnatal day 10 were shown in Figure 7.1. The area under the serum concentration-time curve (AUC) for both oral and IV administration was calculated by the linear trapezoidal method with extrapolation to infinity determined using the ratio of the last serum concentration and elimination rate constant (i.e. C_{last}/k). The elimination rate constant (k) was determined by linear regression analysis of the postdistributive log-linear terminal serum concentration-time determinations. The elimination half-life ($t_{1/2}$) was determined by the ratio $0.693/k$. The systemic clearance (Cl_s) was calculated as $Dose_{IV}/AUC_{\infty}$. Cefepime absolute bioavailability (F) after oral administration was determined by as $(AUC_{Oral} \times Dose_{IV}) / (AUC_{IV} \times Dose_{Oral})$. The apparent volume of distribution (V_d) was determined as $Dose_{IV} / (k \times AUC_{\infty})$.

Calculation of Exposure Indices. The exposure index related to maternal dose (EI_{Dose}) was calculated as:

$$EI_{Dose} = \frac{C_{SS,milk}}{Dose_{Mat}} \left(\frac{M}{S} \right) \left(\frac{V_{milk}}{\tau} \right) \times 100\% \quad (\text{Equation 7.3})$$

where $C_{SS,milk}$ is the steady state drug concentration in rat milk ; $Dose_{Mat}$ is the continuous intravenous infusion dose administered to the dam (mg/hour/kg), M/S is the ratio of steady state milk concentration to steady state serum concentration in the dam. The values for $C_{SS,milk}$, $Dose_{Mat}$, and M/S were obtained from Ling and Alcorn, 2008 (1). V_{milk}/τ is the neonatal milk consumption rate (mL/day), which was obtained from “Rat Breeding Guide”. The exposure index related to maternal concentration (EI_{conc}) was calculated as:

$$EI_{Conc} = \frac{F_{Neo}}{Cl_{Neo}} \left(\frac{M}{S} \right) \left(\frac{V_{milk}}{\tau} \right) \times 100\% \quad (\text{Equation 7.4})$$

where F_{Neo} is the absolute bioavailability in rat pup; Cl_{Neo} is the systemic clearance in the rat neonate (L/h/kg); M/S is the ratio of steady state milk concentration to steady state serum concentration obtained from Ling and Alcorn, 2008 (1); and V_{milk}/τ is the neonatal milk consumption rate (mL/day), which was obtained from “Rat Breeding Guide”(231).

Statistical Analysis. Data is presented as mean \pm SEM. Means between different age groups within the same route of administration were compared using unpaired t-test.

Means for F were compared using unpaired t-test. Significance level was set at 0.05.

7.4 Results

Table 7.1 presents the mean pharmacokinetic parameter estimates for cefepime following oral and intravenous administration to rat pups 4 and 10 days of age. Following IV administration elimination rate constant, half-life and systemic clearance were significantly higher in 10 day-old rat pups relative to postnatal day 4 pups. Although not statistically significant, oral bioavailability was also increased in postnatal day 10 pups. The volume of distribution (V_d) of cefepime was similar between the two rat pup age groups.

Table 7.2 presents additional parameters required for the calculation of Exposure Indices. Note that the cefepime M/S was higher at early lactation stage (day 4) relative to day 10 of lactation (I). At postnatal day 4 the EI_{Dose} and EI_{Conc} were similar and represented about 3% of maternal exposures. The ~7-fold decrease in M/S values of cefepime at day 10 of lactation resulted in ~7-fold reduction in the calculated EI_{Dose} (considering the age-dependent changes in body weight and the volume of milk consumed per day) and ~13-fold reduction in the EI_{Conc} (considering the age-dependent increases in systemic clearance and the volume of milk consumed per day).

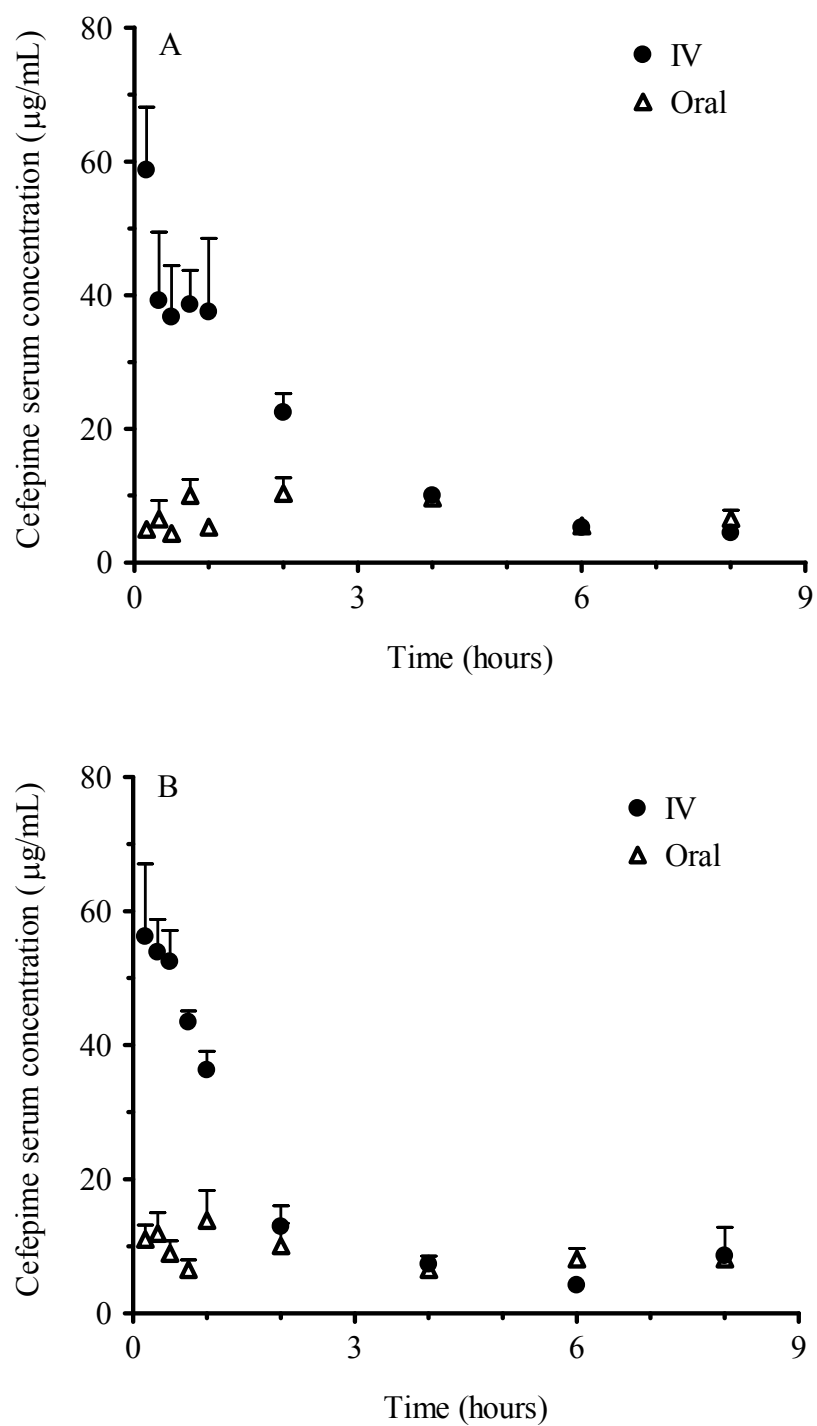


Figure 7.1. Mean \pm SEM serum concentration versus time curves of cefepime after 20mg/kg IV dose or 40mg/kg oral dose in Sprague Dawley rat pups (n=6) at postnatal day 4 (A) and postnatal day 10 (B).

Table 7.1. Summary of mean \pm SEM pharmacokinetic parameters for cefepime after intravenous (20 mg/kg by intracardiac injection under isoflurane anaesthesia) and oral (40 mg/kg by gastric gavage) administration in rat pups (postnatal day 4 and day 10) (n=6/time point blood collection).

Parameters	IV		Oral	
	Day 4	Day 10	Day 4	Day 10
AUC ($\mu\text{g}\times\text{h mL}^{-1}$)	118 \pm 11	101 \pm 9.6	54.1 \pm 7.8	66.3 \pm 14
k (h^{-1})	0.367 \pm 0.02	0.518 \pm 0.03 [#]	0.235 \pm 0.02	0.356 \pm 0.11
Cl (mL/h)	1.68 \pm 0.19	4.26 \pm 0.45 [#]	7.59 \pm 1.68	15.8 \pm 3.8
Cl ($\text{L h}^{-1} \text{kg}^{-1}$)	0.179 \pm 0.02	0.207 \pm 0.02	0.812 \pm 0.11 [*]	0.747 \pm 0.18 [*]
F	-----	-----	0.239 \pm 0.04	0.360 \pm 0.08
t _{1/2} (h)	1.93 \pm 0.13	1.36 \pm 0.08 [#]	3.67 \pm 0.57	3.09 \pm 0.98
V _d (L kg^{-1})	0.506 \pm 0.07	0.404 \pm 0.04	1.33 \pm 0.30 [*]	3.58 \pm 1.6 [*]

IV: Intravenous; AUC: Area-under-the-plasma-concentration-versus-time curve; k: Elimination rate constant; Cl: Clearance; F: Bioavailability; t_{1/2}: Elimination half-life; V_d: Volume of distribution.

Means between different age groups within the same route of administration were compared using unpaired t-test. Means for F were compared using unpaired t-test.

[#] $\alpha < 0.05$.

^{*}Oral clearance and volume of distribution estimated as Cl_S/F and V_d/F, respectively.

Table 7.2. Additional parameters used for the calculation of EI_{Dose} and EI_{Conc} values for postnatal day 4 and day 10 Sprague-Dawley rat pups.

Parameters	Day 4	Day 10
Average Pup Body weight (g)	9.24	21.0
Milk consumption rate (mL/24h) ^a	5	8
Cefepime $C_{SS,milk}$ (mg/mL) ^b	0.394	0.139
Cefepime $Dose_{Mat}$ (mg/kg/h) ^b	250	250
Cefepime M/S ratio ^b	0.89	0.12
EI_{Dose}	0.70%	0.05%
EI_{Conc}	2.64%	0.34%

^aValues obtained from “Rat Breeding Guide” (231).

^bValues obtained from Ling and Alcorn, 2008 (1).

7.5 Discussion

Following maternal exposure to chemicals, exposure indices that relate risk to the nursing neonate as a percentage of maternal dose (EI_{Dose}) or of maternal steady state serum concentration (EI_{Conc}) incorporate milk-to-serum values of the chemical as a significant determinant of the exposure risk (8). For most compounds the literature reports single M/S values and the reported value is assumed to reflect the distributional characteristics of the drug throughout most of the lactation period (232). Our study clearly demonstrates through experimentation that the M/S value of an actively transported chemical changes considerably with stage of lactation, which, in turn, significantly impacts the magnitude of the exposure risk calculated by the EI_{Dose} or EI_{Conc} .

The magnitude of the risk posed to the neonate following exposure to chemicals via the breast milk depends on the amount of drug in the milk and the pharmacokinetic and pharmacodynamic properties of the drug in the neonate (156, 161). Given the proportional relationship between M/S and the EI_{Dose} or EI_{Conc} (see Equations 7.1 and 7.2), M/S estimates have a significant impact on the value of the EI. M/S estimates are

available for some drugs and environmental toxicants, but most, if not all, are determined without regard to lactation stage. The literature does acknowledge differences in M/S between colostrum and mature milk (225, 233) and hind milk versus fore milk (159, 229). However, the breast milk levels of many solutes (i.e. critical nutrients, drugs) also depend upon the activity of SLC and ABC transporters located on the apical and/or basolateral membranes of the polarized lactating mammary epithelium (29, 76). Although the role of most transporters in the mammary gland is largely unknown, recent reports on transporter expression profiles during advancing lactation clearly identify lactation-stage dependent changes in the mRNA and functional expression levels of various transporters (1, 94-95, 234). Changes in expression can result in alterations in the distributional characteristics and extent of accumulation of drugs and environmental toxicants that are substrates for transporters expressed at the lactating mammary gland. Coupling this with the known ontogeny of transporters and other elimination mechanisms in the developing neonate (30-31), the assessment of exposure risk to the nursing neonate can be impacted significantly by postnatal age of the neonate and stage of lactation.

Previously we had reported the acute interaction between the β -lactam antibiotic, cefepime, and L-carnitine transport into rat milk (1). In this study, short-term cefepime intravenous infusion to the rat dam caused a significant decrease in L-carnitine transfer into milk that may be associated with the extent of expression of organic cation/carnitine transporters in the lactating mammary gland. The reduction in L-carnitine milk levels was most significant at early lactation stage (2-fold reduction in milk L-carnitine levels) as compared to mid lactation, which seemed to match a 2-fold higher organic cation/carnitine transporter 2 expression in early lactation and the higher M/S values of cefepime at this lactation stage (0.89 vs 0.12). Both the EI_{Dose} and EI_{Conc} predict a greater risk of exposure to younger neonates given the higher cefepime M/S values at early lactation stage relative to mid-lactation (Table 7.2). The ~7.5-fold difference in the M/S values between day 4 and day 10 of lactation (1) also suggests the Exposure Indices would be over- or underestimated depending upon the stage of lactation at which the M/S value for a given chemical is determined.

In addition to the bioavailable dose to the neonate via the breast milk, neonatal

elimination capacity is an additional critical determinant of exposure risk (8). In the present study, bioavailability was not statistically significantly changed between the two age groups but rat neonates showed an enhanced capacity to eliminate cefepime with age resulting in a considerable decrease in the EI_{Conc} at postnatal day 10 relative to postnatal day 4 (Table 7.2). These data suggest that for a given dose via the breast milk the internal dosimetry metrics (i.e. blood concentrations, area under the blood concentration versus time curve) change with age of the neonate. The potential for drug accumulation and, hence, susceptibility for adverse outcomes may also change with neonatal age. These experimental data confirm the current discourse on the importance of the developmental maturation of pharmacokinetic processes in the assessment of exposure risks during postnatal development.

Our study provides experimental evidence of the importance of lactation stage in the determination of milk-to-serum ratios and brings into question the accuracy of milk-to-serum ratios of some chemicals reported in the literature. This becomes a critical consideration when Exposure Indices are used in the risk/benefit analysis concerning the safety of medication use during breastfeeding or the safety of maternal environmental exposures to a nursing neonate. For drugs, the published Guidances on drug use during breastfeeding (235) significantly impact health care professionals in their considerations of drugs and breastfeeding mothers. Since these Guidances principally focus on drug transfer into breast milk and the amount of drug the neonate becomes exposed to during breastfeeding (8, 102) our study suggests the need to consider lactation stage in the risk/benefit analysis for drugs that are known transporter substrates. What may be safe to use at one stage of lactation may not be considered safe to use at an earlier or later lactation period due to differences in milk-to-serum ratios of a drug. An additional consideration in the risk/benefit analysis is the recent publications that demonstrate the importance of Solute Carrier and ATP Binding Cassette transporter expression at the lactating mammary in nutrient and drug transport into breast milk (1, 90, 94, 236). We recently showed a significant drug-nutrient transport interaction at the lactating mammary gland (1), and our present data suggest the outcomes of a drug-nutrient transport interaction may vary qualitatively and quantitatively at different lactation stages. Furthermore, these new evidences in general are disconcerting because drugs

considered 'safe' during breastfeeding are known to inhibit nutrient transport at other blood-epithelial barriers that have lead to adverse outcomes (237-238) possibly requiring nutritional interventions to alleviate a nutrient deficiency (239-240) . The outcome of drug-nutrient transport interactions at the lactating mammary gland and in the developing neonate following breast milk exposures to drug is relatively poorly investigated.

Chemicals transferred into milk may pose a significant health risk to the nursing neonate. Exposure indices that estimate neonatal drug exposure following maternal exposures to chemicals variably consider the key determinants of risk. Our study confirms the importance of considering the ability of neonate to eliminate an exposed dose due to age-dependent changes in elimination mechanisms as a critical determinant of exposure risk. More importantly, our study experimentally confirms the importance of M/S and the need to consider lactation stage for compounds that are substrates for transporters expressed at the lactating mammary epithelium. For such chemicals an evaluation of M/S at different lactation stages is necessary to avoid an over- or underestimate of the risk when M/S values are assumed to remain constant throughout most of the lactation period. Investigations are needed concerning the role of transporters in the transfer of chemicals across the blood-mammary epithelial barrier, their role in defining M/S, and how the M/S may change with lactation stage as the functional expression of these transporters change with advancing lactation. Such considerations also prompt questions concerning the influence of drugs and other exogenous factors on nutrient transport at the lactating mammary gland and on M/S values.

8 Systematic Evaluation of L-Carnitine Homeostasis Mechanisms during Postnatal Development in Rat

8.1 Abstract

The conditionally essential nutrient, L-carnitine, plays a critical role in a number of physiological processes vital to normal neonatal growth and development. We conducted a systematic evaluation of the developmental changes in tissue organic cation transporter (Octn) expression and free L-carnitine levels, liver γ -butyrobetaine hydroxylase (Bbh) expression and activity, heart carnitine palmitoyltransferase (Cpt) expression and activity, and heart high energy phosphate substrates at different postnatal ages to better understand the interrelationship between these pathways during postnatal development. mRNA expression of Octns, Bbh and Cpts was measured using quantitative real time RT-PCR. Bbh and Cpt activity was measured by validated methods. L-Carnitine levels were determined by HPLC-UV. Both serum and heart L-carnitine levels increased with postnatal development. Increases in serum L-carnitine correlated strongly with postnatal increases in renal Octn2 expression, and was further matched by postnatal increases in intestinal Octn1 expression and hepatic γ -Bbh activity ($P < 0.05$). Postnatal increases in heart L-carnitine levels were strongly correlated to postnatal increases in heart Octn2 expression. Although cardiac ATP levels remained constant through postnatal development, Cr and ADP showed developmental increases with advancing age of the neonate. mRNA levels of Cpt1b and Cpt2 significantly increased at day 20, which was not accompanied by a similar increase in activity. In conclusion, several L-carnitine homeostasis pathways underwent significant ontogenesis during postnatal development in the rat. However, the exact relationship between these pathways and their contribution to L-carnitine homeostasis during development is not completely known and further studies are required to clarify their contributions.

8.2 Introduction

L-Carnitine is a conditionally essential nutrient that functions in a number of physiological processes vital to normal neonatal growth and development (241). With transition to extrauterine life the carbohydrate rich, low-fat umbilical blood supply is replaced by the high fat, low glucose diet of the breast milk. This switch in nutrition source requires physiological adaptations to quickly occur in the newborn to enhance gluconeogenic processes and fatty acid oxidation pathways to meet the energy demands of the tissues (241). L-Carnitine plays an important role in the enhancement of fatty acid utilization and oxidation processes during this adaptive period and throughout development (37, 242). By regulating the movement of long chain fatty acids into mitochondria making them available for β -oxidation, L-carnitine has an obligatory function in cellular energy production. Additionally important is L-carnitine's role in the removal of toxic fatty acyl-CoA metabolites from the mitochondria and maintaining an appropriate balance between free carnitine and its acylated forms (243). Given its vital role in cellular metabolism, increasing evidence points towards the importance of L-carnitine homeostasis in maintaining normal mitochondrial function and in the prevention of disease (243-245). Normal neonatal growth and development, then, may require the maintenance of appropriate L-carnitine homeostasis with postnatal maturation (246).

L-Carnitine homeostasis represents a balance between 1) *de novo* biosynthesis, 2) intestinal absorption from dietary sources, 3) uptake and release by the tissues, and 4) renal reabsorption and excretion (244, 247). During postnatal life, the enzymes and transporters involved in these homeostatic pathways undergo developmental maturation to ensure the requirements of L-carnitine by the developing neonate. Limited information is available on the systematic evaluation of these homeostatic pathways as well as the possible interrelationship between these pathways and L-carnitine levels in the body during postnatal development.

L-Carnitine, a water soluble trimethylated quaternary amine amino acid, is obtained from dietary sources and *de novo* biosynthesis from the amino acids lysine and methionine (247). However, neonates are developmentally immature with regard to L-carnitine biosynthesis due to low levels (approximately 10-12% adult levels) of hepatic

γ -butyrobetaine hydroxylase, the enzyme that mediates the last step in L-carnitine biosynthesis (53, 186, 203). The activity of γ -butyrobetaine hydroxylase (Bbh) increases with age and reaches adult values later in postnatal development (248). Consequently, neonates are highly dependent on dietary sources of L-carnitine.

Dietary L-carnitine is absorbed across the gastrointestinal mucosa by active transport systems at the luminal enterocyte membrane and by passive processes at the basolateral enterocytic membrane (247). Membrane transporters also mediate the tissue distribution and renal reabsorption and excretion of L-carnitine and thus play a critical role in L-carnitine homeostasis. The organic cation/carnitine transporters (OCTN) have been extensively studied for their important roles in L-carnitine transport (198, 249). OCTN1 is a multispecific, bidirectional organic cation transporter that functions in a pH-dependent and sodium-independent manner with wide tissue distribution and low affinity for L-carnitine (190). OCTN2 is the major high affinity sodium-dependent L-carnitine transporter with wide tissue distribution (179, 192). OCTN2 plays a major role in regulating plasma and tissue pools of carnitine in the body (244). OCTN3 is a peroxisomal membrane transporter mediating the intracellular transport of L-carnitine with high specificity (198). OCTN3 has limited tissue distribution with expression identified in the testis, intestine, and kidney (198, 202, 250). These transporters may undergo changes in expression with postnatal maturation, but limited data is available on their ontogeny in different tissues (164, 179, 251).

As major components of the L-carnitine shuttle system, L-carnitine acyltransferase enzyme systems play important roles in fatty acid metabolism and energy production and maintaining an appropriate balance between free and acylated fatty acids (244, 252). Cpt1 on the mitochondrial outer membrane catalyses the first step of mitochondrial import of long chain fatty acids by converting them from fatty acyl-CoA to acylcarnitines (253). Different tissues express different isoforms of Cpt1 (254-255). On the inner mitochondrial membrane Cpt2 reconverts the acylcarnitines to the respective CoA esters releasing free carnitine and making fatty acids available for β -oxidation (256). Limited information is available on the ontogeny of Cpt enzymes.

Given our laboratory's interest in understanding the impact of exogenous influences (i.e. drugs) on the ontogenesis of nutrient homeostasis pathways, particularly

when transporters play a significant role, we first required an understanding of the normal developmental pattern of key processes in the nutrient homeostatic pathway. As a conditionally essential nutrient L-carnitine homeostasis mechanisms undergo a genetic programming to increase utilization of fatty acids during the lactation period and a transporter system plays a key role in maintenance of normal L-carnitine homeostasis. However, the data on L-carnitine homeostatic pathways is limited and any available data typically involved an assessment of the developmental maturation of different processes in isolation of other processes. Hence, we conducted a systemic evaluation of the developmental changes in tissue L-carnitine levels, tissue Octn expression, liver Bbh expression and activity, heart Cpt1 and Cpt2 expression and activity, and heart high energy phosphate compounds at different postnatal ages simultaneously in the same animal with consideration of the known maturation of L-carnitine biosynthesis for the rat (257).

8.3 Materials and Methods

Animals and Chemicals. Female Sprague-Dawley rats ordered at different gestation stages were obtained from Charles River Canada (St. Constant, PQ) and were housed singly in a temperature and humidity controlled facility ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) on a 12-hour light: dark cycle (0700 h – 1900 h). All rats were allowed a 7-day acclimatization period and had free access to food (Prolab® RMH 3000, Purina, Inc., Richmond, IN) and water throughout the study. The dams were closely monitored near parturition to identify the exact time of birth. The dam was considered the experimental unit and blood and tissues were pooled from 5 pups from each dam to carry out the various analyses. Rat pups at postnatal day (PD) 4, 8, 11 and 20 were anaesthetized with isoflurane and blood (200 – 500 uL depending on age) was collected by intracardiac puncture. The rats were immediately sacrificed and heart, intestine, kidney, and liver were rapidly excised and flash-frozen in liquid nitrogen with storage at -80°C until analysis. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethics Board of the University of Saskatchewan.

RNeasy Midi kits were obtained from Qiagen Inc. (Mississauga, ON). The

QuantiTect SYBR Green RT-PCR kit was from Applied Biosystems (Foster City, CA). The Advanced Protein Assay kit was obtained from Fluka (Buchs, Switzerland). L-Carnitine and other chemicals not otherwise specified were obtained from Sigma-Aldrich (St. Louis, MO) and were the highest purity grade available.

L-Carnitine Analysis in Serum and Heart. Serum and heart L-carnitine was quantified by HPLC-UV with pre-column derivatization according to Feng et al (258). Briefly, the pooled heart samples were homogenized with phosphate buffer (50 mM, pH 7.4) in a ratio of 50 mg tissue:250 μ L buffer. The homogenate was centrifuged at 2500 \times g for 10 min at 4°C. The supernatant or serum (20 μ L) sample was precipitated using acetonitrile and methanol (9:1 v/v). A 300 mg mixture of Na₂HPO₄ and Ag₂O (9:1 wt/wt) and 300 mg of KH₂PO₄ were added followed by a 1 h vortex-mixing. Derivatization reagent (40 mg/mL p-bromophenacyl bromide with 50 μ L 40% tetrabutylammonium hydroxide solution) was added into the organic extract. The reaction mixture was incubated at 60°C for 2 h followed by centrifugation at 12,000 \times g for 15 min. L-Carnitine was analyzed using a Hewlett Packard 1050 HPLC system with Diode Array Detector, Quaternary Pump and Autosampler. Samples (10 μ L) were injected onto a CN (cyano) column (HyperClone 5 μ m, 250 \times 4.6 mm, Phenomenex, Torrance, CA) with detection wavelength set at 260 nm. The mobile phase (90% acetonitrile/10 mM citric-phosphate buffer, pH 3) was delivered at a flow rate of 1 mL/min. The standard curve range was linear ($r^2 > 0.99$) between 2.5-40 μ mol/L. Intra- and interassay accuracy and precision ranged from 6%-14%.

Heart Carnitine Palmitoyltransferase (Cpt) Enzyme Activities. Heart Cpt enzyme activities were measured using the spectrophotometric method described by Bieber et al. (259). Briefly, frozen heart tissue was homogenized in 10% (wt/v) homogenization buffer (20 mM HEPES, 140 mM KCl, 10 mM EDTA and 5 mM MgCl₂, pH 7.4) supplemented with 3 mg nagarse using Polytron homogenizer (Brinkmann Instruments, Rexdale, Canada). The homogenate was then centrifuged at 500 \times g for 10 min at 4°C. The supernatant was collected in new tubes and centrifuged at 9000 \times g for 35 min at 4°C. The pellet was then washed with the homogenization buffer without nagarse and centrifuged at 9000 \times g for 35 min at 4°C. The washed pellet was resuspended in 200 μ L homogenization buffer without nagarse. Protein concentrations

were measured using the Advanced Protein Assay kit with bovine serum albumin as standard. The optimal protein concentration and reaction time to give linear product formation were initially determined. To determine total Cpt activity 20 µg protein was assayed in 200 µL ml reaction buffer containing 20 mM HEPES, 1 mM EGTA, 220 mM sucrose, 40 mM KCl, 0.1 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 1.3 mg/mL BSA, and 40 µM palmitoyl-CoA, pH 7.4. The reactions were initiated by adding 1 mM L-carnitine and read at 412 nm after 5 min incubation at 37°C using Synergy HT Multi-Mode Microplate Reader (Biotek instrument, USA). Cpt2 activity was determined using the same reaction conditions as total Cpt except 10µL Cpt1 inhibitor, malonyl-CoA, was added into 200 µL of the reaction mixtures to obtain a final concentration of 10 µM. Cpt1 activity was calculated by subtracting the Cpt2 activity from the total Cpt activity. The Cpt activity was calculated as amount of CoASH released per min per mg protein, which is based on the 5-thio-2-nitrobenzoate formation from CoASH-DNTB reaction. The extinction coefficient for 5-thio-2-nitrobenzoate was 13.6 mM/cm and was used to calculate enzyme activity (Alhomida, 2001; Yu et al., 2001).

Liver γ-Butyrobetaine Hydroxylase (Bbh) Enzyme Activity. The liver tissue was homogenized in homogenization buffer consisting of 300 mM sucrose, 1 mM EGTA, and 50 mM Tris, pH 7.5 using 1:4 mass to volume ratio. The homogenate was then centrifuged at 13,000×g for 30 min at 4°C. The supernatant was collected and centrifuged at 100,000×g for 1h at 4°C. 300 µL of the supernatant representing the cytosolic fraction containing Bbh was then transferred into a dialysis tubing cellulose membrane (molecular weight cutoff of 12,000Da, D9777, Sigma, USA), and submerged in 5 L dialysis buffer (75 mM KCl, 0.1mM DTT and 0.5mM EDTA in sodium phosphate buffer, pH 7.4) overnight in 4°C. The dialyzed sample (20 µL) was tested for L-carnitine residue and the remaining dialysate was stored at -80°C for Bbh testing. The optimal protein concentration, substrate concentration and reaction time to give linear product formation was determined for each age group. For determination of Bbh activity in rat pup liver, 1mL reaction buffer consisting of 0.2 mM γ-butyrobetaine, 20 mM potassium chloride, 3 mM 2-oxoglutarate, 10 mM sodium ascorbate, 0.4 mg/mL catalase in 20 mM potassium phosphate buffer pH 7.0 was prepared. The reaction was initiated by adding 2 µL ferrous ammonium sulfate (133) (final concentration: 0.25 mM) and 20 µL dialyzed

enzyme into 78 μ L reaction buffer and incubated for 25 min at 37°C using a Boekel/Grant Orbital and Reciprocating Water Bath (Model ORS200, Expotech, USA). The reaction was then terminated by adding 10X volume of acetonitrile:methanol (9:1). The mixture was centrifuged at 13,000 \times g for 2 min and the supernatant was used for L-carnitine analysis by HPLC.

Total mRNA Isolation and Quantitative RT-PCR Analysis. Total mRNA was extracted from different tissues using RNeasy Midi Kits (Qiagen Inc., Mississauga, ON) according to manufacturer instructions. RNA purity and quantity was determined spectrophotometrically by measurement at 260 nm and the OD260/OD280 ratio, respectively, with Synergy HT Multi-Mode Microplate Reader (Biotek instrument, USA). Total RNA was stored at -80°C until analysis. Gene sequences were obtained from the National Center for Biotechnology Information GeneBank (260) and specific primers were designed using Primer3 software (Whitehead Institute for Medical Research. Primer 3) (261) (Table 8.1). Quantitative RT-PCR (QRT-PCR) analysis was carried out using a QuantiTect SYBR Green RT-PCR kit and an Applied Biosystems 7300 Real-Time PCR system. The QRT-PCR protocol was carried out according to manufacturer's instructions. The protocol consisted of reverse transcription (1 cycle at 48°C, 30 minutes), PCR initial activation step (1 cycle at 95°C, 15 minutes), three-step thermal-cycling (40 cycles; denaturing at 94°C, 15 seconds, annealing at 60°C, 30 seconds, and primer extension at 60°C for 30 seconds), and a melt curve analysis from 65°C-95°C at 0.5°C/second.

Validation of Primers. Quantitative RT-PCR assays were initially optimized to give PCR efficiency between 1.9-2.1 (as determined by a 3-point standard curving using serial dilutions of control RNA with a slope range of -2.9 to -3.5) and a single melt-peak corresponding to the appropriate PCR product as verified by 2% agarose gel electrophoresis. In addition, the amplification efficiency of each target and β -actin was determined by constructing a standard curve from the crossing point (C_T) value and RNA concentration. The target genes and β -actin were then amplified using the same diluted samples. The ΔC_T values were calculated (i.e. the difference between the target gene C_T and β -actin C_T). The slope from log RNA concentration versus ΔC_T was close to zero (<0.1). Only primers giving PCR amplification close to 100% and the relative

efficiencies between the target and β -actin that were approximately equal were used in our experiment.

Table 8.1. Primer sequences for quantitative RT-PCR of rat enzymes and transporters involved in L-carnitine homeostasis.

Gene	Accession Number	Primers	
		Forward	Reverse
β -actin	NM_031144	agcgtggctacagcttcacc	tgccacaggattccataccc
Octn1	NM_022270	catggctgtgcagactgg	gcaccatgtagccgatgg
Octn2	NM_019269	ggcgcaaccacagtatcc	ggggctttccagtcaccc
Octn3	NM_019723	gacaccgtgaacctgagc	ccatccaggcagttctcc
Cpt1b	NM_013200	cagccatgccaccaagatc	aagggccgcacagaatcc
Cpt2	NM_012930	gctccgaggcggtttctca	tggccgttgccagatagc
Bbh	NM_022629	acgatggggcagagtcc	ctggcctcctgagaaaagc

Octn1, organic cation/carnitine transporter 1; Cpt, carnitine palmitoyltransferase; Bbh, γ -butyrobetaine hydroxylase.

Statistical Analysis. All data are reported as mean \pm SEM. One way ANOVA with Tukey's post hoc test were used for the comparisons between ages. A $P < 0.05$ was chosen as the level of statistical significance. Pearson's correlation coefficients were computed to quantify the association between heart L-carnitine levels and heart Octn2 mRNA expression levels.

8.4 Results

To examine the ontogenesis of critical components of the L-carnitine homeostasis pathway we conducted an mRNA expression and activity analysis of various components of the homeostasis pathway in different tissues of rat pups of different postnatal ages. Compared to postnatally day (PD) 4, serum free L-carnitine concentrations were significantly higher at PD11 without further significant increases thereafter (Figure 8.1A). Heart free L-carnitine levels also increased with postnatal age but showed a significant increase between PD11 and PD20 (Figure 8.1B). Heart and kidney Octn2 expression increased postnatally although increases in expression after postnatal day 11 were not statistically significant (Figure 8.2A and 2B). In the intestine Octn1 expression was low at PD4 but increased rapidly to PD8 and remained elevated throughout the postnatal period. Generally intestinal Octn2 and Octn3 expression was high in the early postnatal period, and expression decreased nonsignificantly through the remaining postnatal period (Figure 8.2C). A reasonable correlation ($r = 0.462$) was observed between serum free L-carnitine concentrations and kidney Octn2 mRNA expression ($P < 0.05$) in rat pups at different postnatal age groups. In addition, heart free L-carnitine concentrations were strongly correlated ($r = 0.823$) to heart Octn2 mRNA expression ($P < 0.05$) in rat pups at different postnatal age groups (Figure 8.3). Liver Bbh mRNA expression and activity increased postnatally although increases beyond PD11 were not statistically significant (Figure 8.4). Heart Cpt1b and Cpt2 mRNA expression remained constant in the early postnatal period with significant increases in expression occurring only between PD11 and PD20 (Figure 8.5). Despite this increase in mRNA expression in the late postnatal period no significant differences in activity were found at all examined postnatal ages (Figure 8.6). In addition, heart ATP levels remained constant through postnatal development (Figure 8.7). The concentrations of cardiac Cr increased significantly during postnatal development (Figure 8.7). Furthermore, our data also showed good correlation between ADP and postnatal age in rat pups (Figure 8.7).

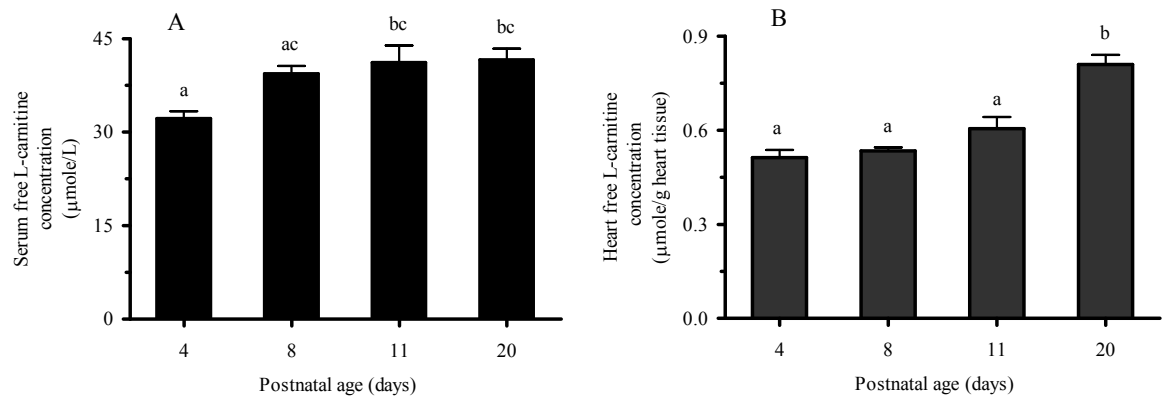


Figure 8.1. Mean \pm SEM Free L-carnitine levels in rat pup serum (A) and heart (B) (n=6) at different postnatal ages. Bars with the same letters indicate no significant difference, $P < 0.05$.

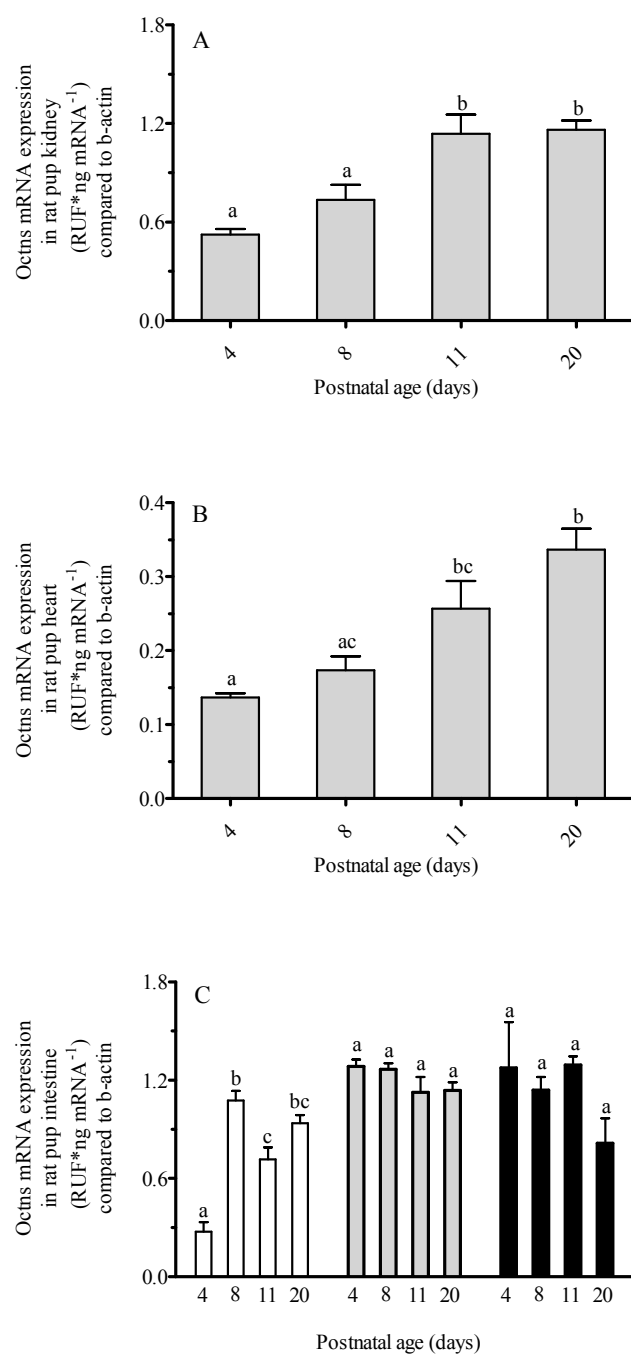


Figure 8.2. Mean \pm SEM Organic cation/carnitine transporter (Octn1 – white bar; Octn2 – light grey bar; Octn3 – dark grey bar) mRNA expression levels in rat pup heart (A), kidney (B), and intestine (C) at different postnatal ages (n=6). mRNA expression was normalized to β -actin. Bars with the same letters indicate no significant difference, $P < 0.05$.

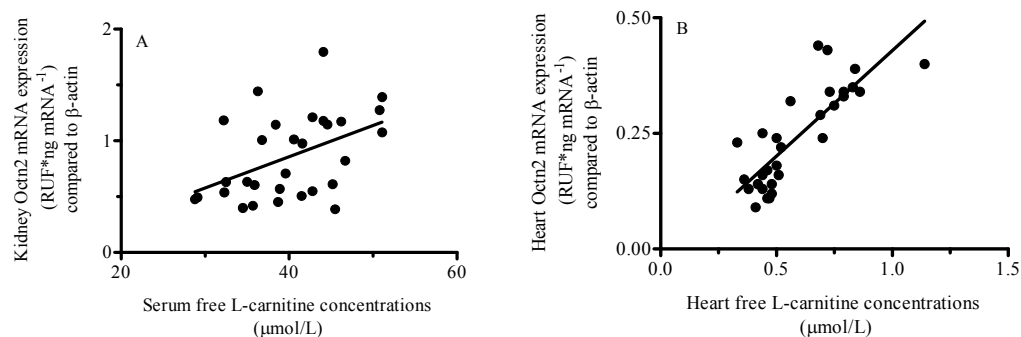


Figure 8.3. A: Correlation between kidney Octn2 mRNA expression levels and serum free L-carnitine concentration ($r=0.462$). B: Correlation between heart Octn2 mRNA expression levels and heart free L-carnitine concentration ($r=0.823$).

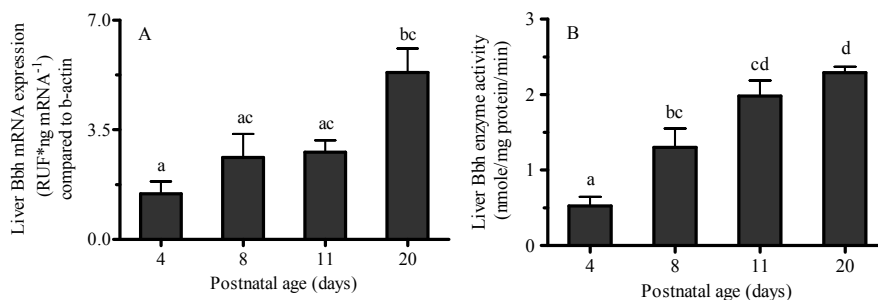


Figure 8.4. Mean \pm SEM liver gamma-butyrobetaine hydroxylase (Bbh) mRNA expression level (A) and activity (B) in rat pups at different postnatal age groups ($n=6$). mRNA expression was normalized to β -actin. Bars with the same letters indicate no significant difference, $P<0.05$.

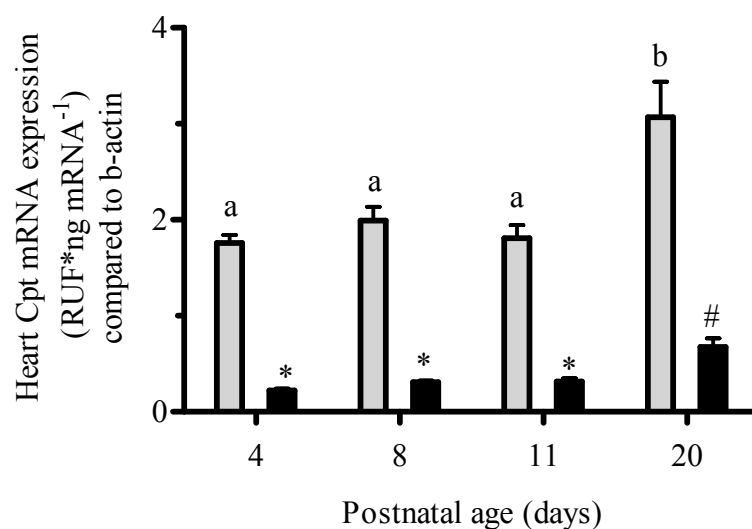


Figure 8.5. Mean \pm SEM mRNA expression level of heart carnitine palmitoyltransferase 1b (Cpt1b) (light grey bar) and carnitine palmitoyltransferase 2 (Cpt2) (black bar) in rat pups at different postnatal age groups (n=6). Expression was normalized to β -actin. Bars with the same letters (mRNA expression levels) or same symbol (activity levels) indicate no significant difference, $P < 0.05$.

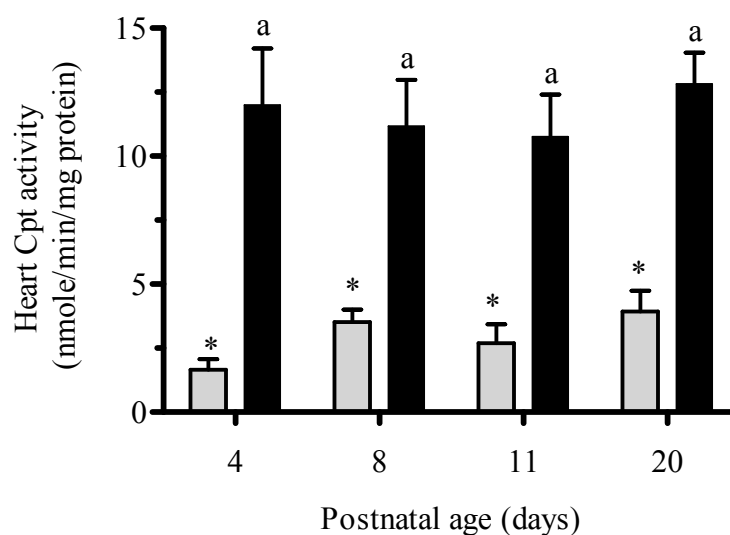


Figure 8.6. Mean \pm SEM heart carnitine palmitoyltransferase 1 (Cpt1) (light grey bar) carnitine palmitoyltransferase 2 (Cpt2) activity in rat pups at different postnatal age groups (n=6). Bars with the same symbol (Cpt1) or same letters (Cpt2) indicate no significant difference, $P < 0.05$.

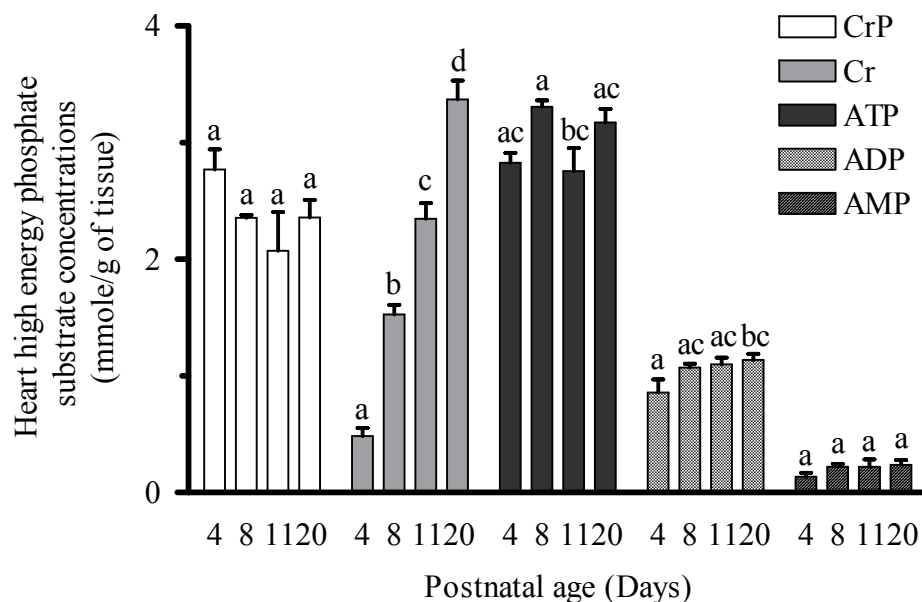


Figure 8.7. Mean \pm SEM heart high energy phosphate substrate concentration in rat pups at different postnatal age groups (n=6). Bars with the same letter indicate no significant difference, $P < 0.05$.

8.5 Discussion

During postnatal development, a number of nutrient homeostasis pathways undergo ontogenesis to satisfy the nutrient requirements for growth and development. The developmental changes in these nutrient homeostasis pathways are usually genetically programmed. However, these processes can be affected by exogenous factors such as dietary components, environmental compounds and therapeutic drugs. Since we are interested in understanding how drug-nutrient transport interactions may impact the ontogeny of important nutrient homeostatic processes, we systemically evaluated L-carnitine homeostasis pathways in the rat, since L-carnitine levels in the body critically depend on transporter function in the gastrointestinal tract and the kidney (180). The information on the ontogeny of L-carnitine homeostasis pathways can help us to further understand the impact of exogenous factors on their maturation and its possible long

term consequences.

In mammals, fatty acid oxidation becomes the main source of energy for many tissues with transition to extrauterine life (37, 174). Mitochondrial fatty acid utilization, though, requires a sufficient supply of L-carnitine to shuttle long-chain fatty acids across the mitochondrial membrane making them available for β -oxidation (183). The maternal circulation supplies L-carnitine to the developing fetus. During late gestation, L-carnitine concentrations significantly increase in fetal tissues and this storage of L-carnitine assures adequate levels in the immediate postpartum period (262). These tissue stores become quickly depleted due to the immaturity of many of the L-carnitine homeostasis mechanisms. In our study, postnatal increases in serum free L-carnitine was consistent with levels reported in the literature (125) and these increases correlated with maturation of a number of enzymes and transporter systems that critically determine L-carnitine levels in the body.

Lower serum free L-carnitine levels in the early postnatal period is, in part, due to the limited capacity for endogenous biosynthesis by the young neonate (53, 186, 257). As noted in other studies, we found that hepatic γ -Bbh mRNA expression and activity was significantly lower at early postnatal development in rat pups (257, 263). Young neonates are highly dependent on exogenous sources of L-carnitine, which is usually supplied in sufficient amounts by the breast milk during nursing (187). Interestingly, L-carnitine levels in the milk of nursing rat dams decrease significantly by mid-lactation (1, 182). Despite the reduced exogenous L-carnitine, serum free L-carnitine levels in rat pups increase with advancing age (125, 185). The maturation of hepatic γ -Bbh contributes to the postnatal increase in L-carnitine levels in the body. However, our study also suggests that maturation of other processes, namely renal reabsorption of L-carnitine additionally contribute to the postnatal rise in serum L-carnitine.

Absorption of dietary sources of L-carnitine requires the function of several transporter systems expressed at the gastrointestinal epithelial barrier (174). Expression of duodenal Octn2 and Octn3 did not change with postnatal development. This is not consistent with increases in Octn2 expression noted in the literature (264) but could be the result of different rat strains (Sprague Dawley vs. Wistar) or different portion of the small intestine (jejunum or ileum vs. duodenum). Both Octn2 and Octn3 mediate active

uptake of L-carnitine (198, 265). Lack of changes in mRNA expression with development may suggest that the absorptive capacity of L-carnitine by these transporter systems remains unchanged. However, without protein expression levels and measurements of transport activity with postnatal development, definitive conclusions are difficult to make. Interestingly, the mRNA expression level of duodenal Octn1 was significantly increased during development. Octn1 is a low affinity, bidirectional L-carnitine transporter with ubiquitous expression and plays an important role in the tissue availability of L-carnitine (189-191). However, whether the postnatal increase in Octn1 expression in the intestine contributes to an enhanced absorption of L-carnitine from dietary sources requires clarification.

Renal reabsorption of L-carnitine from the urinary filtrate plays a significant role in maintenance of L-carnitine levels in the body. Almost 95% of the excreted L-carnitine is reabsorbed by transporters expressed in the proximal tubules of the kidney with Octn2 as the principal transporter involved in this process (179). In our study renal Octn2 expression increased during postnatal development in the rat, which is consistent with the literature (179, 251). The increase in renal Octn2 expression correlated strongly with increases in serum L-carnitine levels suggesting that renal Octn2 plays a significant role in the postnatal pattern of serum L-carnitine development. Overall our data suggests the developmental changes in hepatic γ -Bbh expression, intestinal Octn1 expression, and renal Octn2 expression may systemically contribute to the postnatal increase in serum L-carnitine levels. However, the precise interconnections of these pathways and their overall contribution to L-carnitine homeostasis during development is not known and further studies are required to clarify their contributions.

The distribution of L-carnitine in the body is organ dependent with the highest concentration of L-carnitine in the heart (266). In our study, heart L-carnitine levels increase during postnatal development and these increases were correlated with increased expression of Octn2 in the heart. L-Carnitine has a significant role in energy production in neonatal cardiac tissue due to its role in fatty oxidation and the reliance of neonatal hearts on fatty acids as the primary energy substrate (184). The dramatic increase in fatty acid oxidation rates in early heart development after birth has been attributed to an increase in L-carnitine levels (184). Although we observed a significant

increase in L-carnitine levels in the heart with advancing age of the neonate, cardiac ATP levels remained constant through postnatal development. Interestingly, we found that Cr and ADP levels were ontogenically regulated during postnatal development. The significance of such developmental changes is not clear and requires investigation.

We also evaluated heart Cpt enzyme expression and activity due to the pivotal role of these enzymes in heart energy production. The postnatal increase in both Cpt1b and Cpt2 mRNA expression at postnatal day 20 are paralleled by the increases in heart L-carnitine concentrations. Indeed, Cpt1a and Cpt2 mRNA levels were increased by carnitine administration in cell culture systems (267). Thus, the significant increase in heart L-carnitine levels at postnatal day 20 may account for the transcriptional enhancement of both Cpt1b and Cpt2. Despite these transcriptional increases in Cpt enzymes, we observed no significant changes in heart Cpt enzyme activity. Cpt enzyme activities have been reported to increase with increasing mitochondrial L-carnitine levels (268-270). Unfortunately, L-carnitine levels in the whole heart tissue rather than in the mitochondria were measured in our study.

In conclusion, several L-carnitine homeostasis pathways underwent significant ontogenesis during postnatal development in the rat. However, the exact relationship between these pathways and their contribution to L-carnitine homeostasis during development is not completely known and further studies are required to clarify their contributions. Such a clarification is necessary to understand the impact of exogenous and endogenous factors on L-carnitine status during development. Nonetheless, this systematic evaluation of key pathways in the L-carnitine homeostasis pathway provides a basis from which we can conduct further evaluations regarding the effects of exogenous and endogenous factors on L-carnitine status during postnatal development and possible long-term consequences of any disturbance in the normal ontogeny of these pathways.

9 Drug-Nutrient Transport Interaction during Ontogeny: The Cefepime L-Carnitine Example

9.1 Abstract

Our study investigated the potential for an important *in vivo* drug-nutrient transport interaction in developing neonates using the L-carnitine transporter substrates, cefepime and L-carnitine, as proof-of-concept. Rat pups were administered cefepime (5mg/kg) twice daily by subcutaneous injection according to different dosing schedules (postnatal day 1-4, day 1-8, day 8-11, day 8-20 and day 1-20). Cefepime administration significantly reduced serum and heart free L-carnitine levels in groups which started treatment early and were of short duration (day 1-4, day 1-8 and day 8-11) ($P < 0.05$). Severe degenerative changes in ventricular myocardium were also found in these groups. Cefepime treatment also altered the ontogeny of several key mechanisms in the L-carnitine homeostasis pathway. The qualitative and quantitative changes in levels of γ -butyrobetaine hydroxylase mRNA and activity, intestinal organic cation/carnitine transporter (Octn) mRNA, and renal Octn2 mRNA depended on time and duration of cefepime exposure. Despite lower levels of heart L-carnitine in earlier postnatal groups, levels of carnitine palmitoyltransferase mRNA and activity, heart Octn2 mRNA and ATP levels in all treatment groups remained unchanged with cefepime exposure. However, changes in other high energy phosphate substrates (ADP, AMP and CrP) were noted and reductions in the CrP/ATP ratio were found in rat pups with normal serum L-carnitine levels. In summary, our data suggest a significant *in vivo* drug-nutrient transport interaction in the developing neonates which can lead to detrimental consequences depending on the time and duration of the exposure. In addition, specific windows of susceptibility towards a drug nutrient transport interaction may exist during postnatal development.

9.2 Introduction

Nutrient homeostasis pathways and processes governing drug pharmacokinetics in neonates undergo ontogenesis. When a drug and nutrient share the same mechanism, a significant drug-nutrient interaction during postnatal development could affect the ontogeny of these pathways. Neonatal illness often requires a pharmacological intervention to reduce the burden of disease-associated morbidity and mortality. However, most investigations into drug-nutrient interactions examine the influence of nutrients on drug pharmacokinetics and/or pharmacodynamics (239, 271-273). Despite a general acknowledgement that drug use may impact the normal development of the physiological system, limited research has examined the long-term consequences of pharmacological interventions on the developing neonate, particularly when drugs influence nutrient availability in the body (274).

Early postnatal life represents a period of significant susceptibility to nutritional alterations that may affect maturing biochemical and physiological processes with long term consequences on individual health (59, 275). Developing individuals can adapt to nutritional changes to favor survival (276-277). These nutritional adaptations can be subtle or obvious, reversible or irreversible, and transient or permanent depending on factors such as the stage of physiological development, duration of nutritional alteration and whether the alteration occurs during a critical window of susceptibility (278-279).

Many nutrients require transporter systems to make them available to the physiological system and cellular processes (83, 280-281). During postnatal development, nutrient transporters undergo significant ontogenesis that reflects a developmental need for nutrients required for growth and metabolism. A number of these nutrient transporter systems also mediate drug transfer across epithelial barriers and cell membranes (282-283). A requirement for the same transporter system could result in potential reversible interactions between a drug and a nutrient that may alter nutrient absorption and disposition in the developing individual. At present, no research is available on the potential risk of drug-nutrient transporter interactions that cause nutritional alterations in the neonates. Yet the magnitude and significance of a putative drug-nutrient transporter interaction in a neonatal patient could vary depending on the size and duration of a drug exposure and the timing of that exposure during postnatal

development. Such factors may lead to different adaptive strategies to optimize nutrient homeostasis in a neonate and may consequently lead to significant changes in neonatal development especially when the nutrient affected is critical for neonatal development. An important question, then, is whether pharmacological interventions in the neonate can result in a significant drug-nutrient transporter interaction that alters the maturing biochemical and physiological processes involved in drug absorption/disposition processes and nutrient homeostasis.

In a proof-of-concept investigation, we used the conditionally essential nutrient, L-carnitine, and the β -lactam antibiotic, cefepime, both known to be substrates for carnitine transporters expressed at epithelial barriers and cellular membranes. Maintenance of L-carnitine levels is important for normal mitochondrial and endothelial functions, crucial for normal neonatal growth and development (245, 257). During postnatal life, the major pathways involved in L-carnitine homeostasis undergo significant ontogenesis (164, 251, 257). The organic cation/carnitine transporter 2 (Octn2) is particularly important in the maintenance of L-carnitine homeostasis (55, 179, 189). The substrate specificity of Octns also includes a variety of therapeutically used cationic drugs such as the β -lactam antibiotics (219, 284). Both *in vitro* and *in vivo* research has identified significant competitive interactions between such drugs and L-carnitine (1, 219).

In acknowledgement of the paucity of information regarding drug-nutrient interactions during early postnatal life we used the known L-carnitine and cefepime competitive transporter interaction to identify possible alterations in the ontogeny of L-carnitine homeostasis pathways. We also examined whether the consequences of a drug-nutrient transporter interaction depends on the timing and duration of drug exposure time relative to postnatal maturation. The overall purpose of our study was to provide the first experimental evidence of metabolic programming as a result of a significant drug-nutrient transporter interaction during postnatal development.

9.3 Materials and Methods

Animals, Diet, and Chemicals. Female Sprague-Dawley rats ordered at gestation day 16 were obtained from Charles River Canada (St. Constant, PQ) and were

housed singly in a temperature and humidity controlled facility ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) on a 12-hour light: dark cycle (0700 h – 1900 h). All rats had free access to food and water throughout the study and were allowed a 7-day acclimatization period. All procedures were conducted in accordance with the Canadian Council of Animal Care guidelines for the care and use of laboratory animals and were approved by the Animal Care and Supply Committee of the University of Saskatchewan. Throughout the acclimatization period and during the study, rats had available *ad libitum* a rat diet (Prolab® RMH 3000, Purina, Inc., Richmond, IN) (protein % = 22.5; fat, ether extr. % = 4.5; fat, acid hydr. % = 6.4; crude fiber % = 4.0; ash % = 6.1; calcium % = 1.0; phosphorous % = 0.75) meeting the nutritional requirements for lactating Sprague-Dawley rats.

Cefepime was purchased from the Royal University Hospital at the University of Saskatchewan (Saskatoon, SK). RNeasy Midi kits were obtained from Qiagen Inc. (Mississauga, ON). The Advanced Protein Assay kit was obtained from Fluka (Sigma-Aldrich, MO), QuantiTect SYBR Green RT-PCR kit was purchased from Applied Biosystem (Foster City, CA). L-carnitine and other chemicals not otherwise specified were obtained from Sigma-Aldrich (St. Louis, MO).

Chronic Cefepime Administration in Rat Pups. Cefepime (5 mg) or saline (control) was subcutaneously administered to rat pups (n=6 dams per dosing schedule) twice daily using the following schedules: 1) postnatal day 1 to day 4; 2) postnatal day 1 to day 8; 3) postnatal day 1 to day 20; 4) postnatal day 8 to day 11; and 5) postnatal day 8 to day 20. These dosing schedules considered short-term and long-term cefepime treatment and developmental maturation of L-carnitine biosynthesis pathways in rat pups (postnatal day 8) (Galland et al., 1999; Huhn et al., 1981). Total body weights were recorded daily. At the end of each dosing schedule, blood, heart, intestine, kidney and liver were collected. All tissues were snap frozen in liquid nitrogen and stored at -80°C . Blood and tissues were pooled from 5 pups from each mother such that the dam was considered the experimental unit.

Free L-Carnitine Analysis in Serum and Heart. Serum and heart free L-carnitine was quantified by HPLC-UV with pre-column derivatization according to Feng et al (258). Briefly, the pooled heart samples were homogenized with phosphate buffer (50 mM, pH 7.4) in a ratio of 50 mg tissue: 250 μL buffer. The homogenate was

centrifuged at 2500×g for 10 min at 4°C. The supernatant or serum (20 µL) sample was precipitated using 9:1 acetonitrile: methanol in a 1:9 v/v. A 300 mg mixture of Na₂HPO₄ and Ag₂O (9:1 wt/wt) and 300 mg of KH₂PO₄ were added followed by 1 h vortex-mixing. Derivatization reagent (40 mg/mL p-bromophenacyl bromide with 50 µL 40% tetrabutylammonium hydroxide solution) was added into the organic extract. The reaction mixture was incubated at 60°C for 2 h followed by centrifugation at 12,000×g for 15 min. L-Carnitine was analyzed using a Hewlett Packard 1050 HPLC system with Diode Array Detector, Quaternary Pump and Autosampler. Samples (10 µL) were injected onto a CN (cyano) column (HyperClone 5 µm, 250 × 4.6 mm, Phenomenex, Torrance, CA) with detection wavelength set at 260 nm. The mobile phase (90% acetonitrile/10% mM citric-phosphate buffer, pH 3) was delivered at a flow rate of 1 mL/min. The standard curve range was linear ($r^2 > 0.99$) between 2.5-40 µmol/L. Intra- and interassay accuracy and precision ranged from 6%-14%.

Total mRNA Isolation and Quantitative RT-PCR Analysis. Total mRNA was extracted from different tissues using RNeasy Midi Kits according to manufacturer instructions. RNA purity and quantity was determined spectrophotometrically by measurement at 260 nm and the OD260/OD280 ratio, respectively, using a Synergy HT Multi-Mode Microplate Reader (Biotek instrument, USA). Total RNA was stored at -80°C until analysis. Gene sequences were obtained from the National Center for Biotechnology Information GeneBank (260) and specific primers were designed using Primer3 software (261) (Table 9.1). Quantitative RT-PCR (QRT-PCR) analysis was carried out using a QuantiTect SYBR Green RT-PCR kit and an Applied Biosystems 7300 Real-Time PCR system. The QRT-PCR protocol was carried out according to manufacturer's instructions. The protocol consisted of reverse transcription (1 cycle at 48°C, 30 minutes), PCR initial activation step (1 cycle at 95°C, 15 minutes), three-step thermal-cycling (40 cycles; denaturing at 94°C, 15 seconds, annealing at 60°C, 30 seconds, and primer extension at 60°C for 30 seconds), and a melt curve analysis from 65°C-95°C at 0.5°C/second.

Validation of the 2^{-ΔΔCT} Method. Real-time PCR assays were initially optimized to give PCR efficiency between 1.9-2.1 (as determined by a 3-point standard curving using serial dilutions of control RNA with a slope range of -2.9 to -3.5) and a single

melt-peak corresponding to the appropriate PCR product as verified by 2% agarose gel electrophoresis. The reactions were further optimized for usage of the $2^{-\Delta\Delta C_T}$ method using β -actin as an internal standard. The amplification efficiency of each target and β -actin was determined by constructing a standard curve from crossing point values (C_T) and RNA concentration. The target genes and β -actin were then amplified using the same diluted samples. The ΔC_T were calculated (i.e. the difference between the target gene C_T and β -actin C_T). Only primers giving PCR amplification close to 100% and the relative efficiencies between the target and β -actin that were approximately equal were used in our experiment (i.e. the slope from log RNA concentration versus ΔC_T were <0.1). Fold differences in mRNA expression between control and treated samples were then calculated.

Table 9.1. Primer sequences for quantitative RT-PCR of all the selected targets

Gene	Accession Number	Primers	
		Forward	Reverse
B-actin	NM_031144	Agcgtggctacagcttcacc	Tgccacaggattccataccc
Octn1	NM_022270	Catggctgtgcagactgg	Gcaccatgtagccgatgg
Octn2	NM_019269	Ggcgcaaccacagtatcc	Ggggctttccagtcaccc
Octn3	NM_019723	Gacaccgtgaacctgagc	Ccatccaggcagttctcc
Cpt1b	NM_013200	Cagccatgccaccaagatc	Aagggccgcacagaatcc
Cpt2	NM_012930	Gctccgagcggtttctca	Tggccgttgccagatagc
Bbh	NM_022629	Acgatggggcagagtcc	Ctggcctcctgagaaaagc

Octn, organic cation/carnitine transporter 1; Cpt, carnitine palmitoyltransferase 1b; Bbh, γ -butyrobetaine hydroxylase.

Liver γ -Butyrobetaine Hydroxylase (Bbh) Enzyme Activity. Liver tissue was homogenized in homogenization buffer consisting of 300 mM sucrose, 1 mM EGTA, and 50 mM Tris, pH 7.5 using 1:4 mass to volume ratio. The homogenate was then centrifuged at 13,000 \times g for 30 min at 4°C. The supernatant was collected and centrifuged at 100,000 \times g for 1h at 4°C. 300 μ L of the supernatant representing the cytosolic fraction containing Bbh was then transferred into a dialysis tubing cellulose membrane (molecular weight cutoff of 12,000Da, D9777, Sigma, USA), and submerged in 5 L dialysis buffer (75mM KCl, 0.1mM DTT and 0.5mM EDTA in sodium phosphate buffer, pH 7.4) overnight in 4°C. The dialyzed sample (20 μ L) was tested for L-carnitine residue and the remaining dialysate was stored at -80°C for Bbh testing. The optimal protein concentration, substrate concentration and reaction time to give linear product formation was determined for each age group. For determination of Bbh activity in rat pup liver, 1 mL reaction buffer consisting of 0.2 mM γ -butyrobetaine, 20 mM potassium chloride, 3 mM 2-oxoglutarate, 10 mM sodium ascorbate, 0.4 mg/mL catalase in 20 mM potassium phosphate buffer pH 7.0 was prepared. The reaction was initiated by adding 2 μ L ferrous ammonium sulfate (133) (final concentration: 0.25 mM) and 20 μ L enzyme into 78 μ L reaction buffer and incubated for 25 min at 37°C using a Boekel/Grant Orbital and Reciprocating Water Bath (Model ORS200, Expotech, USA). The reaction was then terminated by adding 10X volume of acetonitrile: methanol (9:1). The mixture was centrifuged at 13,000 \times g for 2 min and the supernatant was used for L-carnitine analysis by HPLC.

Heart Carnitine Palmitoyltransferase (Cpt) Enzyme Activities. Heart Cpt enzyme activities were measured using the spectrophotometric method described by Bieber et al (259). Briefly, frozen heart tissue was homogenized in 10% (wt/v) homogenization buffer (20 mM HEPES, 140 mM KCl, 10 mM EDTA and 5 mM MgCl₂, pH 7.4) supplemented with 3 mg nagarse using Polytron homogenizer (Brinkmann Instruments, Rexdale, Canada). The homogenate was then centrifuged at 500 \times g for 10 min at 4°C. The supernatant was collected in new tubes and centrifuged at 9000 \times g for 35 min at 4°C. The pellet was then washed with the homogenization buffer without nagarse and centrifuged at 9000 \times g for 35 min at 4°C. The washed pellet was

resuspended in 200 μ L isolation buffer without nagarse. Protein concentrations were measured using the Advanced Protein Assay kit with bovine serum albumin as standards. The optimal protein concentration and reaction time to give linear product formation were initially determined. To determine total Cpt activity 20 μ g protein was assayed in 200 μ L ml reaction buffer containing 20 mM HEPES, 1 mM EGTA, 220 mM sucrose, 40 mM KCl, 0.1 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 1.3 mg/mL BSA, and 40 μ M palmitoyl-CoA, pH 7.4. The reactions were initiated by adding 1 mM L-carnitine and read at 412 nm after 5 min incubation at 37°C using Synergy HT Multi-Mode Microplate Reader (Biotek instrument, USA). Cpt2 activity was determined using the same reaction conditions as total Cpt except 10 μ L Cpt1 inhibitor, malonyl-CoA, was added into 200 μ L of the reaction mixtures to obtain a final concentration of 10 μ M. Cpt1 activity was calculated by subtracting the Cpt2 activity from the total Cpt activity. The Cpt activity was calculated as amount of CoASH released per min per mg protein, which is based on the 5-thio-2-nitrobenzoate formation from CoASH-DNTB reaction. The extinction coefficient for 5-thio-2-nitrobenzoate was 13.6 mM/cm (Alhomida, 2001; Yu et al., 2001).

Heart High Energy Phosphate Substrate Determination. The heart high energy phosphate substrate levels including creatine (Cr), creatine phosphate (CrP), ATP, ADP and AMP were measured with HPLC-UV method as described by Olkowski et al., (285). The heart samples were homogenized in 0.7 M ice cold perchloric acid (MW 100.46) with a final concentration 100 mg/ml. The homogenate was centrifuged at 12,000 rpm for 5 minutes. The supernatant was collected and neutralized with 2M potassium hydroxide to bring pH near to 7.0. The supernatant was then filtered through 0.45 μ m filter (Nonsterile Syringe Filter Nylon, Chromatographic Specialties Inc. Brockville, Ontario, Canada) and 10 μ L was injected into a 3 μ Luna C-18 (Phenomenex, Torrance, CA) column using gradient flow conditions. Two mobile phase components used included 20 mM potassium phosphate buffer (pH 7.0) and 100% methanol. The gradient was 100% phosphate buffer from 0–6.5 min, 100% methanol from 6.5–12.5 min, and 100% phosphate buffer from 12.5 to 25 min for column re-equilibration, which was sufficient to achieve stable baseline conditions. The high energy phosphate substrates including CrP, Cr, ATP, ADP and AMP were monitored at 210 nm. The standard curve

range was from 6.25-100 µg/mL and the limit of detection was 0.078125 µg/mL for ATP and 0.3125 µg/mL for ADP and AMP. Intra- and interassay accuracy and precision ranged from 4.2% to 14.5%.

Histopathology of the Heart. Heart tissue collected in formalin was embedded in paraffin. The tissue was then sectioned into 5µm thick and stained with hematoxylin and eosin. The histological preparations of hearts (six replications per slide) were subjected to detailed microscopic evaluation. For semi-qualitative analysis, the observed lesions were graded according to *a priori* established standards, taking into consideration histopathological features characteristic of cell degeneration such as cytoplasmic eosinophilia, chromatin condensation and nuclear pyknosis, spongy degeneration of the cytoplasm, cytoplasmic vacuolization, and cariorhexis. The assessment of the degree of myocardial changes was based on arbitrarily set criteria as follows: 1) ‘No visible lesions’ was assigned when all structures of the myocardial tissue appeared morphologically normal, 2) ‘Mild lesions’ was assigned when changes were predominantly focal or multifocal, but limited to a very small area of myocardium, 3) ‘Moderate lesions’ was assigned when changes were focal, but affecting a large area of myocardium, predominantly prominent multifocal, or smaller scale locally extensive, and 4) ‘Severe lesions’ was assigned when changes were multifocal and affecting larger areas of the ventricular myocardium, or were predominantly locally extensive. In order to ensure unbiased evaluation, two pathologists examined slides with a double blind approach (i.e. neither identity of the subjects nor treatments applied were known to the scientists who conducted the evaluation).

Statistical Analysis. All data are reported as mean ± SEM. All experimental data were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The interactions between treatment and duration time for all the selected parameters were assessed by 2x2 factorial ANOVA using General Linear Model (GLM) multivariate. Multiple comparisons for all the parameters at different ages were analyzed using one way ANOVA with Fisher's least significant difference (LSD) as post hoc test. The effects of cefepime on rat pups in each duration period were analyzed by one way ANOVA. In addition, Pearson's correlation coefficients were computed to quantify the association between all the processes involved in maintaining L-carnitine homeostasis (serum free

L-carnitine levels) during development. Data used for this purpose include serum L-carnitine levels, mRNA expression level of kidney Octn2, intestinal Octns, and Bbh mRNA expression and activity in control rats from postnatal day 4, day 8, day 11 and day 20.

9.4 Results

Body Weight Gain. Cefepime caused no differences in total body weight gain or growth pattern regardless of dosing schedule ($P>0.05$) (data not shown). However, cefepime did cause changes in various molecular, biochemical and histological markers depending upon timing and/or duration of cefepime administration relative to postnatal age.

L-Carnitine Levels. Cefepime caused significant decreases in serum L-carnitine levels when rat pups were treated from day 1-4, day 1-8 and day 8-11 ($P<0.05$) with no differences in the other dosing schedule groups ($P>0.05$) (Figure 9.1A). Cefepime caused significant decreases in heart L-carnitine levels when rat pups were treated from day 1-8 and day 8-11 ($P<0.05$) with reduced L-carnitine levels from day 1-4 ($P=0.07$) and no differences in the other groups ($P>0.05$) (Figure 9.1B).

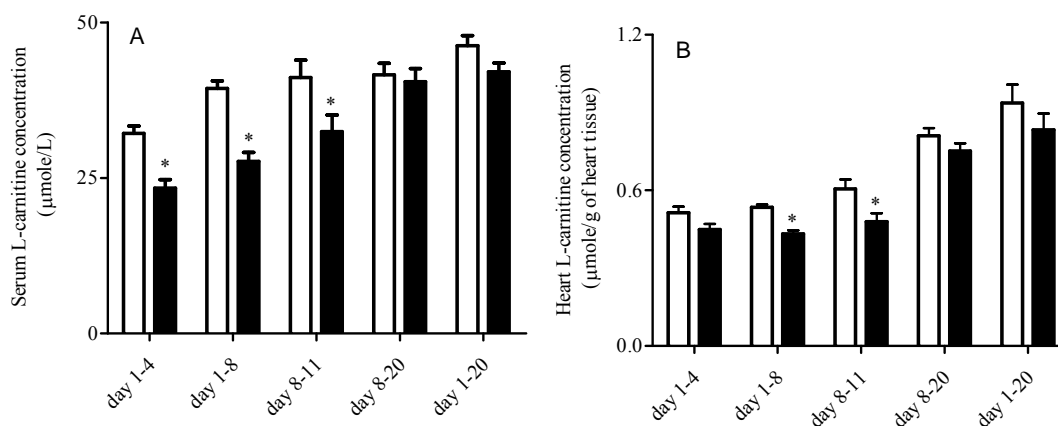


Figure 9.1. Mean \pm SEM free L-carnitine levels in serum (A) and heart (B) from rat pups treated with saline (white bar) or 5 mg cefepime (black bar) twice a day by subcutaneous injection according to different dosing schedules ($n=6$). Means were compared by one way ANOVA with LSD as post hoc analysis; $*\alpha=0.05$.

mRNA Expression Levels of L-Carnitine Transporters. Cefepime treatment caused no difference in heart Octn2 expression ($P>0.05$) (Table 9.2). Kidney Octn2 mRNA expression levels were significantly upregulated in treatment groups from day 1-4, day 8-20 and day 1-20 ($P<0.05$) with no difference in the other groups ($P>0.05$) (Table 9.2). The mRNA expression of Octn2 was significantly upregulated in the intestine after cefepime treatment from day 8-11 ($P<0.05$) with no differences in other treatment groups (Table 9.2). Cefepime treatment caused significant increase in intestinal Octn1 mRNA expression levels in treatment groups day 8-11 and day 1-20 ($P<0.05$) (Table 9.2). The mRNA expression levels were also enhanced by cefepime exposure in treatment groups day 1-8 and day 1-20 (Table 9.2).

Table 9.2. Mean \pm SEM fold differences relative to control in mRNA expression level of L-carnitine transporters (Octns) in kidney, heart and intestine, liver Bbh and heart Cpts from rat pups treated with 5 mg cefepime or saline (control) twice daily by subcutaneous injection according to different dosing schedules (n=6).

	Day 1-4	Day 1-8	Day 8-11	Day 8-20	Day 1-20
Kidney Octn2	1.27 \pm 0.10*	1.17 \pm 0.15	0.98 \pm 0.14	2.24 \pm 0.37*	1.24 \pm 0.05*
Intestinal Octn1	0.99 \pm 0.09	0.90 \pm 0.09	1.89 \pm 0.33*	0.84 \pm 0.08	1.80 \pm 0.16*
Intestinal Octn2	1.18 \pm 0.28	1.06 \pm 0.16	1.22 \pm 0.10*	1.09 \pm 0.17	0.76 \pm 0.21
Intestinal Octn3	1.39 \pm 0.50	1.84 \pm 0.26*	1.02 \pm 0.17	2.21 \pm 0.89	1.51 \pm 0.12*
Heart Octn2	0.99 \pm 0.03	0.88 \pm 0.13	0.95 \pm 0.14	1.09 \pm 0.12	0.92 \pm 0.10
Liver Bbh	1.15 \pm 0.56	0.90 \pm 0.26	0.89 \pm 0.20	0.73 \pm 0.22	0.90 \pm 0.33
Heart Cpt1b	1.02 \pm 0.17	1.03 \pm 0.15	1.34 \pm 0.22	1.00 \pm 0.13	0.83 \pm 0.09
Heart Cpt2	1.19 \pm 0.12	0.97 \pm 0.07	1.32 \pm 0.16	1.15 \pm 0.18	1.10 \pm 0.09

Expression was normalized to β -actin and fold difference was determined using the comparative C_T method for relative quantitation. Means between control and cefepime treatment were compared using unpaired t-test; * $\alpha=0.05$.

Liver Bbh and Heart Cpt Enzyme Expression and Activity. Cefepime caused no changes in Bbh mRNA expression levels in all treatment groups ($P>0.05$) (Table 9.2). However, Bbh enzyme activities were significantly upregulated in rat pups treated from day 8-20 ($P<0.05$). In addition, Bbh enzyme activities were higher in rat pups treated from day 8-11 though the changes were not statistically significant ($P=0.06$) (Figure 9.2A). Cefepime caused significant increases in heart Cpt1b mRNA expression levels (Table 9.2) and Cpt2 activities in rat pups treated from day 1-20 ($P<0.05$) with no effect on other treatment groups (Figure 9.2B and 9.2C).

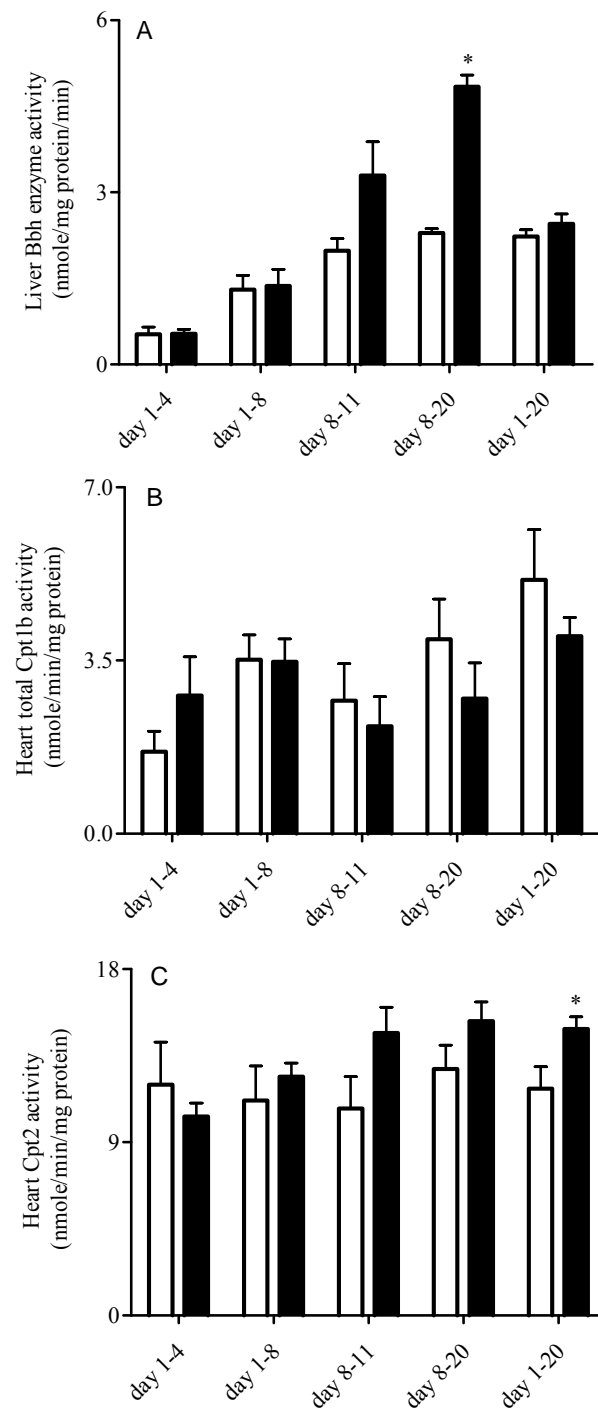


Figure 9.2. Mean \pm SEM activities of liver BbH (A), heart Cpt1 (B) and heart Cpt2 (C) in rat pups treated with saline (white bar) or 5 mg cefepime (black bar) by twice daily subcutaneous injection according to different dosing schedules (n=6). Means were compared using one way ANOVA with LSD as post hoc analysis; * α = 0.05.

Heart High Energy Phosphate Substrate Levels. Heart high energy phosphate substrate profiles in rat pups including ATP, ADP, AMP, creatine and creatine phosphate were measured (Figure 9.3). Creatine phosphate concentration in the rat hearts was reduced by cefepime treatment from postnatal day 8-20 and day 1-20 ($P<0.05$). ADP levels were significantly increased in treatment group postnatal day 1-8 ($P<0.05$) with some increase in day 8-20 ($P=0.08$) and day 1-20 ($P=0.08$). In addition, AMP levels were significantly increased in treatment group postnatal day 1-8 but decreased in treatment group day 1-20 ($P<0.05$). The ratio between CrP and ATP (CrP/ATP ratio) were significantly reduced in treatment group postnatal day 8-20 and day 1-20 ($P<0.05$).

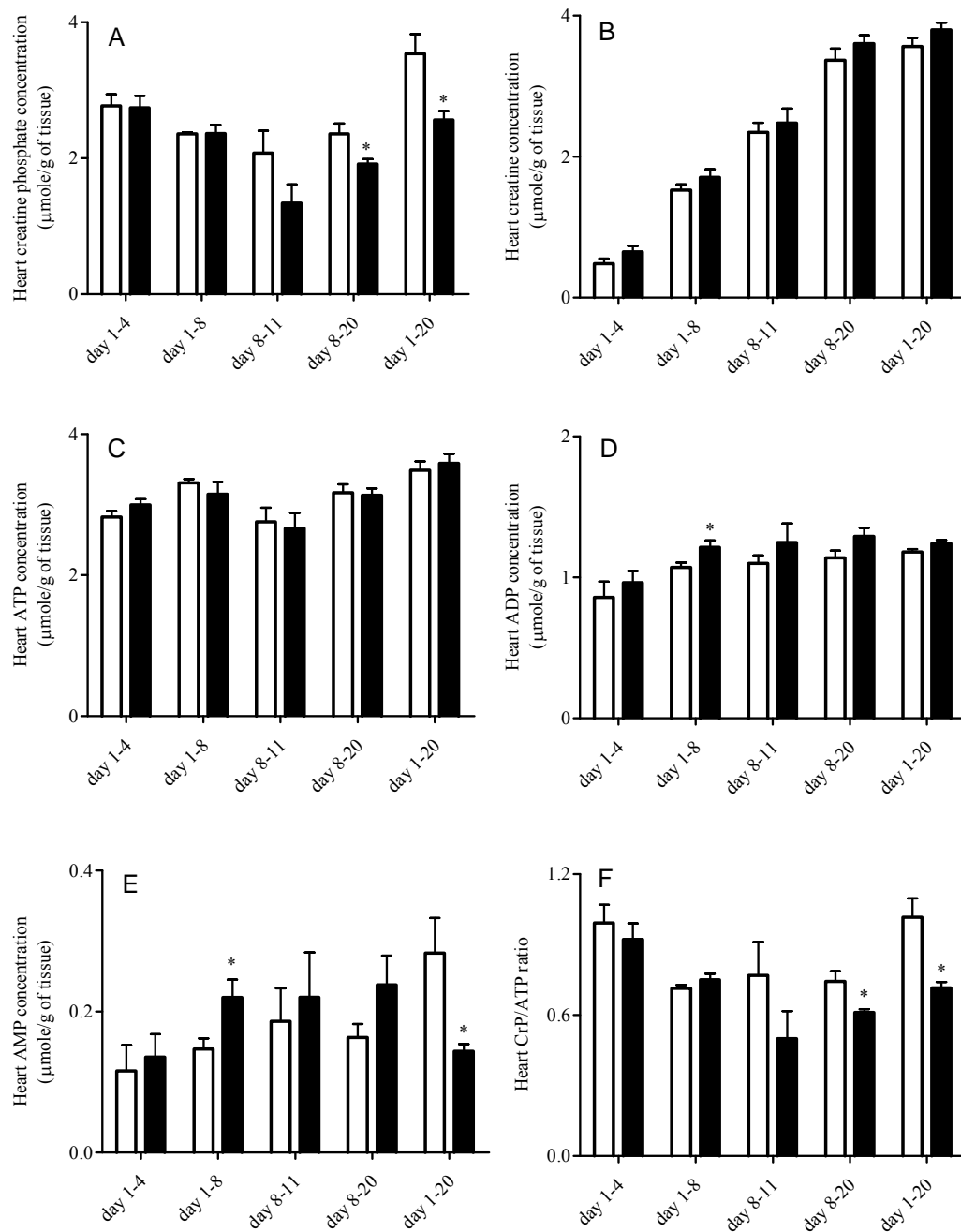


Figure 9.3. Heart high energy phosphate substrate profiles in rat pups treated with saline (white bar) or 5 mg cefepime (dark grey bar) twice daily by subcutaneous injection according to different dosing schedules (n=6). A) creatine phosphate; B) creatine; C) ATP; D) ADP; E) AMP; F) CrP/ATP ratio. Means were compared with one way ANOVA with LSD as post hoc analysis; * $\alpha=0.05$.

Histological Analysis. The heart specimens from both control and treated animals showed some pathological changes indicative of myocardial tissue degeneration. However, the lesions were more severe in myocardium of rat pups treated with cefepime from day 1-4, day 1-8 and day 8-11 with similar lesions in rat pups treated from day 8-20 and day 1-20 (Table 9.3). Representative examples of severe changes are shown in Figure 9.4. Noteworthy are early degenerative changes marked by cytoplasmic eosinophilia and nuclear pyknosis (black arrows). The affected cardiomyocytes had a distinct dull dark pink appearance, and had lost their typical striation pattern. Some cardiac cells showed aggregation of the nuclei. A more advanced pathological process in the cardiomyocytes was evidenced by cariorhexis (blue arrows). Many cells showed spongy degeneration of the cytoplasm, and in some instances cytoplasmic vacuolization was prominent (red arrows). The myocardial syncytium was punctuated by clear spaces between the myofibers and interstitial edema was present in some areas. Cardiomyocyte dropout was evident in more severely affected areas (green arrows) (Figure 9.4).

Correlation Analysis. Most of the L-carnitine homeostasis pathways and high energy phosphate substrates were ontogenically regulated ($P < 0.05$) except liver Bbh mRNA expression, heart CrP levels and heart Cpt2 activities in control animals ($P > 0.05$) (Table 9.4). Cefepime treatment changed the maturation patterns of intestinal Octn2 and Octn3 mRNA expression levels, concentrations of CrP, ATP and AMP, and Cpt1 activities ($P > 0.05$) (Table 9.4).

Interactions between Treatment and Duration Time. There were significant interactions between treatment and duration times on the serum L-carnitine levels, liver Bbh activities and heart AMP levels ($P < 0.05$).

Table 9.3. Mean \pm SEM scores of cardiac lesions in rat pups treated with saline (Control) or 5 mg cefepime (Treated) by twice daily subcutaneous injection according to different dosing schedules (n=6).

	Day 1-4	Day 1-8	Day 8-11	Day 8-20	Day 1-20
Control	1.7 \pm 0.5	2.2 \pm 0.3	1.2 \pm 0.4	1.3 \pm 0.4	1.0 \pm 0.5
Treated	2.5 \pm 0.3	2.8 \pm 0.2	2.2 \pm 0.3	1.2 \pm 0.4	0.3 \pm 0.3
P value	0.240	0.132	0.093	0.937	0.394

Means between control and cefepime treatment were compared using Mann Whitney test; $\alpha = 0.05$. The histopathological score scheme used in this study was: No visible lesion: 0; Mild lesion: 1; Moderate lesion: 2; and Severe lesion: 3.

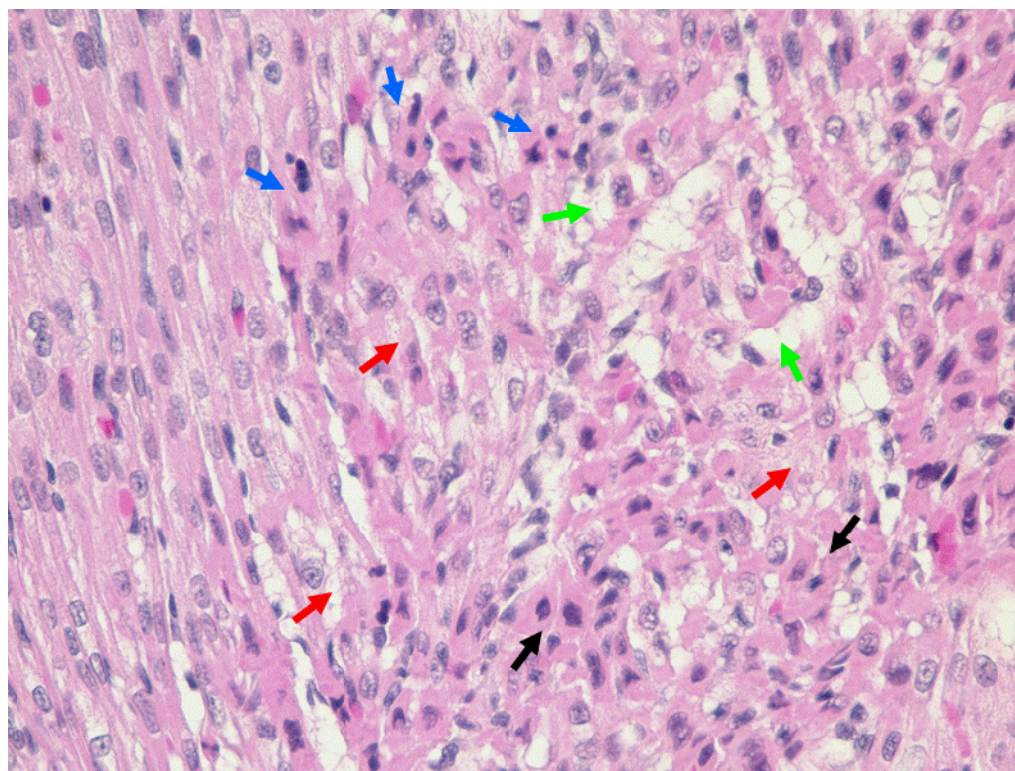


Figure 9.4. Representative histopathological features of the mural ventricular myocardium of rat pup heart (the specimen is from rat pup treated with cefepime from day 1-8). The significance of the arrows and arrowheads is described in the results section of the text (original magnification 400x).

Table 9.4. Pearson's correlation coefficients between postnatal ages and all parameters assessed in rat pups treated with saline (Control) or 5mg cefepime (Treated) by twice daily subcutaneous injection (rat pups were from day 4, day 8, day 11 (n=6 for each age) and day 20 (n=12).

Parameters	Control Animals		Treated Animals	
	Pearson Correlation Coefficient	P value	Pearson Correlation Coefficient	P value
Serum L-carnitine	0.642	0.000	0.861	P<0.0001
Heart L-carnitine	0.840	P<0.0001	0.846	P<0.0001
Kidney Octn2 mRNA	0.369	0.045	0.665	P<0.0001
Heart Octn2 mRNA	0.827	P<0.0001	0.879	P<0.0001
Intestinal Octn1 mRNA	0.305	0.101	0.370	0.044
Intestinal Octn2 mRNA	-0.465	0.010	0.083	0.663
Intestinal Octn3 mRNA	0.803	P<0.0001	0.098	0.608
Liver Bbh activities	0.417	0.022	0.707	P<0.0001
Liver Bbh mRNA	0.034	0.857	0.387	0.035
CrP	0.248	0.186	-0.168	0.375
Cr	0.956	P<0.0001	0.956	P<0.0001
ATP	0.421	0.021	0.358	0.052
ADP	0.533	0.002	0.419	0.021
AMP	0.415	0.023	0.093	0.627
Cpt1 activities	0.564	0.001	0.268	0.152
Cpt2 activities	0.086	0.653	0.603	0.000
Heart Cpt1b mRNA	0.692	P<0.0001	0.382	0.037
Heart Cpt2 mRNA	0.838	P<0.0001	0.901	P<0.0001

9.5 Discussion

Neonates can be exposed to drugs therapeutically and through secondary exposure (eg. via breast milk). When drug exposures are intentional, dosage regimens consider the developmental changes in drug pharmacokinetic processes in pediatric patients with dosage adjustments made according to maturation of enzymes and transporters involved in drug absorption and disposition (286). Some of these pharmacokinetic processes that undergo developmental maturation are also utilized by various nutrients in the body. In particular, a variety of transporters have been identified as having substrate specificities that include both nutrients and therapeutically used drugs (282). Thus, co-administration of a drug may cause a functional interaction with the transporters responsible for nutrient absorption and/or disposition. Knowing that the body may adapt strategies to maintain optimal nutrient homeostasis in the face of nutritional imbalance (287-288), such an interaction has the potential to change nutrient homeostasis pathways in developing neonates. If the interaction occurs during a susceptible window of vulnerability the interaction may result in metabolic programming of the nutrient homeostasis pathway(s), the consequences of which may impact an individual's risk for development of disease (33).

The present study used the conditionally essential nutrient, L-carnitine, and a known inhibitor of L-carnitine transport, cefepime, to examine possible alterations in the ontogeny of L-carnitine homeostasis pathways. This study demonstrated that cefepime administration altered L-carnitine homeostasis pathways in neonatal rats. Of particular note was the dependence upon duration and timing of exposure relative to postnatal development on the qualitative and quantitative changes observed with cefepime exposure. Serum and heart free L-carnitine levels were reduced in groups which started treatment early and lasted shorter (day 1-4, day 1-8 and day 8-11). In contrast, L-carnitine levels remained within developmentally normal levels when rat neonates were treated with cefepime until weaning (day 1-20, and day 8-20) (Figure 9.1). Since L-carnitine is an obligatory nutrient for neonatal development, reduced levels early in postnatal development as a result of the cefepime-L-carnitine interaction may pose a detrimental risk to the neonates. The literature has drawn an association between reductions in L-carnitine levels and development of cardiac hypertrophy and eventually

cardiomyopathy (289). In our study, histopathological examinations revealed severe degenerative changes in ventricular myocardium in the groups with reduced L-carnitine levels. Although minor histopathological changes were identified in groups with normal free L-carnitine blood levels and rats pups treated with saline, these changes were likely attributed to the stress of twice daily injections. The severe degenerative changes in the younger neonates with reduced L-carnitine levels may eventually lead to compromised heart function and a predisposition to cardiovascular disease in later life.

When alterations in nutrient availability occur, the body may have a variety of adaptive responses to maintain nutrient homeostasis. L-Carnitine homeostasis is maintained primarily through endogenous biosynthesis in the liver, exogenous dietary sources, and renal reabsorption of filtered L-carnitine (180). Transporters mediate both the gastrointestinal absorption and active reabsorption of L-carnitine from the urinary filtrate and such transporters account for more than 75% of body L-carnitine (174). In the adult, renal L-carnitine transport systems are known to rapidly adapt to dietary L-carnitine levels through modifications in L-carnitine reabsorption efficiency at the kidneys (290). Our results suggest that neonates also have the capacity to adjust their renal L-carnitine reabsorption by changing Octn2 expression levels, the principle transporter involved in the active reabsorption of L-carnitine from the urinary filtrate (179). Renal Octn2 mRNA expression was highly upregulated in the rat pups demonstrating normal L-carnitine levels despite cefepime treatment (day 8-20 and day 1-20) (Table 9.2). Since Octn2 accounts for more than 95% of L-carnitine reabsorption from the filtrate (179), the increase in Octn2 expression may significantly contribute to the maintenance of L-carnitine levels in these two groups despite competitive interactions with cefepime at renal Octn2.

Duration of treatment also seemed to influence the extent to which renal Octn2 expression increased with cefepime treatment. Limited or no changes in expression was observed with short-term cefepime treatment. Although renal Octn2 mRNA expression was also increased in treatment group postnatal day 1-4, the enhanced expression was not adequate to assure sufficient reabsorption of L-carnitine in the presence of a competitive interaction with cefepime since serum L-carnitine levels were reduced.

In addition to renal Octn2, cefepime also influenced intestinal Octn expression, but the qualitative and quantitative changes varied depending on the treatment schedules. Alterations in intestinal Octn expression may be the result of an adaptive response to altered L-carnitine levels following a competitive interaction with cefepime. Intestinal transporters are known to undergo adaptive regulation according to nutrient status (291-293). Although a competitive interaction at gastrointestinal L-carnitine transporters in rat pups is unlikely following subcutaneous administration, a competitive interaction at renal reabsorption mechanisms may lead to reduced L-carnitine levels and a need to alter intestinal L-carnitine transporters in response to altered L-carnitine status. Interestingly, cefepime administration did not influence heart Octn2 expression in any of the postnatal groups evaluated. These data suggest that regulation of heart Octn2 expression is refractory to the interaction between cefepime and L-carnitine unlike Octn2 expression in the kidney and intestine.

Endogenous biosynthesis of L-carnitine, particularly by the liver, accounts for 25% of L-carnitine in the adult. However, immaturity of hepatic γ -butyrobetaine hydroxylase (Bbh) activity at birth limits the ability of neonates to biosynthesize L-carnitine. In the rat, Bbh activity reaches adult capacity by postnatal day 8 (257). Interestingly, our study demonstrated that hepatic Bbh activity was affected only when cefepime treatment was instituted after Bbh maturation (i.e. day 8). Enhancements in Bbh activity in treatment groups postnatal day 8-20 ($P < 0.05$) and day 8-11 ($P = 0.06$) may be an adaptive response to compensate for the loss of L-carnitine in the body via competitive interactions at renal Octn2. Cefepime exposure did not alter Bbh maturation when treatment was initiated in early postnatal life. These observations may suggest that the maturation of hepatic Bbh activity is genetically programmed and may not be influenced by either internal (i.e. L-carnitine concentration) or external factors (i.e. cefepime exposure). This phenomenon has been noted for other nutrient homeostasis pathways, such as in the case of age related changes in monosaccharide uptake rate, which undergoes genetically programmed maturation and is only slightly influenced by dietary manipulations (294).

L-Carnitine plays an obligate role in energy production as it facilitates the transport of long chain fatty acids across the mitochondrial membrane making them

available for β -oxidation (174, 295). Tissues with low L-carnitine levels often have low fatty acid oxidation rates (296). Despite reductions in serum and heart L-carnitine levels in some treatment groups in our study, Cpt activity and heart Octn2 expression was not altered and cefepime exposure did not influence ATP levels in rat pup hearts. However, alterations in other high energy phosphate substrates including ADP, AMP and CrP were observed depending on the treatment schedules. Unfortunately, the specific interrelationships between the changes in these high energy phosphate substrates are difficult to rationalize in our study. However, we did identify significant decreases in CrP and the CrP/ATP ratios in treatment groups with normal L-carnitine levels (Day 8-20 and day 1-20). These findings are significant because both the loss of creatine phosphate and the reduction in the ratio of CrP/ATP has been associated with several pathological conditions in animals (297). Moreover, the reduction of CrP/ATP ratios has been used as a predictor of mortality for patients with dilated cardiomyopathy (298), which is a typical symptom of L-carnitine deficiency (206). The decreased CrP levels and CrP/ATP ratios may be early signs of heart dysfunction in these cefepime treated animals despite the fact that serum and heart L-carnitine levels were maintained in the normal range.

For many physiological and biochemical processes, considerable maturation of these processes continues throughout neonatal development (163). In the rat, many components involved in the L-carnitine homeostasis pathway undergo significant ontogeny in the period from birth to weaning (Chapter 8). In this study, treatment of rat pups with cefepime, a known inhibitor of L-carnitine transport, altered various L-carnitine homeostasis mechanisms during postnatal development. The qualitative and quantitative changes in these pathways depended upon when during postnatal development the neonate was exposed to the drug, as well as to the duration of the exposure. However, despite compensatory changes in renal and intestinal L-carnitine transport systems, rat pups treated at postnatal day 1-4, 1-8 and 8-11 could not maintain serum and heart L-carnitine concentrations at levels associated with their untreated counterparts. As well, these pups showed more severe histopathological changes in the heart relative to their untreated counterparts and to older treated pups. These data suggest that although young neonates have the ability to mount an adaptive response to

exogenous factors that influence nutrient status, this adaptive response may not be sufficient depending upon the magnitude of the environmental insult. Our data also generally showed that neonates exposed to cefepime later in postnatal development or throughout the postnatal period seemed to mount an adaptive response that was sufficient to maintain serum and tissue L-carnitine levels and prevent pathological changes in the heart.

In conclusion, our study, which was premised on the known competitive interaction between cefepime and L-carnitine at L-carnitine transporter systems, demonstrated that cefepime administration to neonatal rat pups caused significant alterations in the ontogenesis of several mechanisms involved in the L-carnitine homeostasis pathway. These alterations likely represented adaptive responses to cefepime-induced alteration in L-carnitine status. The qualitative and quantitative changes in these L-carnitine homeostasis pathways seemed to depend upon duration and timing of exposure relative to postnatal maturation, but whether these changes are permanent are unknown. Future studies are planned to assess the potential for metabolic programming of L-carnitine homeostasis mechanisms following a cefepime-L-carnitine transport interaction and the long-term consequences of this interaction on the risk for disease later in life. Nonetheless, our findings could have major implications with respect to drug treatment of paediatric patients, particularly young neonates, and highlights a potential need for nutritional interventions during drug therapy in this population.

10 Lipopolysaccharide and Lipoteichoic Acid Differentially Alter Glucose, Fatty Acid and Carnitine Transporter Expression in MCF12A Cells

10.1 Abstract

We investigated whether gram-positive (Lipoteichoic acid, LTA) and gram-negative (Lipopolysaccharide, LPS) bacterial inflammatory stimuli differentially affect glucose, fatty acid and carnitine transporter expression in human mammary epithelial cells. MCF12A cells were incubated with 1 μ g/mL LPS or LTA for 6 or 12 hours and expression of tumor necrosis factor- α (TNF- α), organic cation/carnitine transporters (OCTN1, OCTN2), facilitated glucose transporters (GLUT1, GLUT8) sodium dependent glucose transporter 1 (SGLT1), fatty acid transporters (FATP1, FATP4), and fatty acid binding protein 3 (FABP3) was measured using Quantitative RT-PCR. We quantified high-energy phosphate substrates (ATP, ADP, AMP) by HPLC and oxygen consumption rates using the Hansatech oxygen electrode. LPS decreased expression of OCTN1, OCTN2, FATP1, and FABP3, but increased GLUT1, GLUT8 and SGLT1 expression after 6 hours. Transporter expression returned to control levels or exceeded control (OCTN2, GLUT1, FATP1, FABP3) after 12 hours. LTA caused limited changes with 6 hour exposure but significantly downregulated GLUT1, GLUT8 and SGLT1 and upregulated OCTN1, FATP1 and FABP3 expression after 12 hours. ATP, ADP, and AMP levels were unaltered; however, LTA significantly decreased oxygen consumption rates after 12 hours. LPS and LTA exposure caused qualitative and quantitative differences in the time course and expression of glucose, fatty acid, and L-carnitine transporters in MCF12A cells. Whether changes in transporter expression levels lead to differences in substrate availability to support cellular energy metabolism and/or synthesis of milk constituents warrants further investigation. The time-dependent and reciprocal regulation of glucose transporters relative to carnitine and fatty acid transporters also warrant investigation.

10.2 Introduction

Mastitis is a common condition of breastfeeding mothers (137, 299-300) with reported incidence rates of up to 33% in the United States (Barbosa-Cesnik et al., 2003; Betzold, 2007). Infective mastitis represents an important cause of inflammation of the mammary gland (301) and typically follows from infections with *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram positive) (302-303). These pathogens elicit different pathophysiological outcomes in the mammary gland. Infection induced by *Escherichia coli* is usually acute with extensive damage, but usually resolves within a few days (304). On the other hand, *Staphylococcus aureus* infections may result in a less severe, but chronic and persistent condition (141).

Much research into infective mastitis has focused on the immunological factors that lead to pathogenesis and recovery of the mammary gland from infection. Interestingly, investigations involving other epithelial barriers are uncovering a role for insufficient epithelial cell energy metabolism resulting from decreased availability and utilization of preferred energy substrates as an underlying cause for the pathogenesis of chronic inflammation (305-306). Carbohydrate and lipid are the principal fuels for cellular energy production (307) and these energy substrates gain access to the epithelial cells via transporter systems (76).

During lactation, enhanced ATP generation is necessary to satisfy the energy requirements for milk synthesis and secretion in the mammary gland (308). Principally, glucose and, in part, fatty acids are the major energy substrates for ATP production (113, 308-310). In the lactating mammary gland, transporters play a decisive role in making available the substrates that support mammary epithelial cell energy metabolism, synthetic functions, and various milk components (i.e. lactose, fat, micronutrients) (2, 76, 311). The lactating mammary gland expresses a variety of transporters (76, 83, 95, 234), but our understanding of the influence of exogenous and endogenous factors on their function is limited.

As the principal substrate for ATP generation and synthesis of various milk components, the lactating mammary gland requires the transport of free glucose into the epithelium (312). Two different types of glucose transporter families mediate glucose

uptake and transfer across epithelial barriers: facilitative glucose transporters (GLUTs) and sodium dependent glucose transporters (SGLTs) (75, 85). Both GLUT1 and SGLT1 are expressed in lactating human mammary epithelial cells (84, 313) with GLUT1 accounting for a significant portion of mammary gland glucose transport (309, 313). In addition, GLUT8 was also identified in lactating bovine mammary gland as a high-affinity glucose transporter with moderate expression (85, 314). Free fatty acid availability in the lactating mammary epithelium depends on both *de novo* biosynthesis and uptake from maternal circulation (116). Fatty acid uptake pathways include the fatty acid binding proteins (FABPs) located in the plasma membrane, fatty acid translocators and fatty acid transport proteins (FATPs) (86, 315). FABP3 is the most abundant isoform in the bovine mammary gland (316), and though limited information is available on the expression of FATPs in the human mammary gland, the human term placenta expresses both FATP1 and FATP4 (317). In addition to glucose and fatty acids in ATP generation in epithelial barriers, L-carnitine has critical importance in fatty acid oxidation (318). The lactating mammary gland expresses two members of the organic cation/carnitine transporter family, OCTN1 and OCTN2, which contribute to the transport of L-carnitine into the epithelial cell (1, 83).

Clearly, changes in such transporter levels with inflammation may affect energy substrate availability to the lactating mammary gland. Investigations undertaken with other organ systems or cell types have uncovered intriguing data that suggests inflammation alters transporter expression profiles thereby modulating substrate availability to these tissues (147-148, 319-325). Some studies even correlate inflammation-mediated alteration in transporter expression and activity to organ dysfunction. For example, inflammation downregulates renal tubule glucose transporters, which leads to glucosuria and acute renal failure (148). In the intestine, inflammation decreases L-carnitine transport with reductions in fatty acid oxidation and concomitant loss of mucosal cell number, compromised barrier function, and mucosal ulceration (326-327). Such data suggests inflammatory stimuli may alter the expression of such energy substrate transporters in the mammary gland, which, in turn, may affect substrate availability, cellular metabolism, and biosynthetic capacity in the mammary epithelium.

In this study, we investigated the influence of inflammatory stimuli on the

expression of critical energy substrate transporters of human mammary epithelial cells (GLUT1 and 4, SGLT1, FATP1 and 4, FABP3 and OCTN1 and 2). We used lipopolysaccharide (LPS) and lipoteichoic acid (LTA) as two widely applied model bacterial challenges of mammalian cells (131) to explore the possible differential effects of gram positive and gram negative bacterial stimuli on mRNA expression levels of these transporters. Since the origin of the infectious stimulus is known to cause different pathophysiological outcomes in the mammary gland, this comparison was necessary to support future investigations into whether differential changes in transporter expression profiles could contribute to the different clinical presentations of mastitis caused by gram negative and gram positive pathogens. Finally, quantification of high energy phosphate substrates (ATP, ADP, and AMP) and oxygen consumption rates accompanied the expression analysis to provide functional measures of the consequences of altered transporter expression profiles on cellular energy metabolism.

10.3 Materials and Methods

Chemicals, Reagents and Supplies. Falcon tissue culture flasks (T-75), bottle-top filters (0.2 μ m), and sterile polystyrene 12-cell culture dishes were purchased from VWR (Mississauga, ON, Canada). ATP, ADP, and AMP were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). CellTiter-Glo® Luminescent Cell Viability Assay kit was obtained from Promega (Promega, WI, USA). All other chemicals and solvents were of the highest analytical grade. All water solutions were made from high quality water processed by steam distillation followed by particle absorption, ionic exchange and bacterial filtration by a Milli-Q Synthesis Water System (Millipore, MA, USA).

Cell Culture and LPS/LTA Treatment. The human mammary epithelial cell line (MCF12A) was purchased from American Type Culture Collection (ATCC, Rockville, MD) at passage 52. The cells were cultured, as specified by ATCC, in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 5% horse serum, supplemented with 20 ng/mL human epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL bovine insulin and 500 ng/mL hydrocortisone. The cells were maintained under an atmosphere of 95% air-5% CO₂ at 37°C. Media was changed every 2–3 days.

MCF12A cells were grown in T-75 cell culture flasks and passaged once before LPS/LTA challenge experiments (passage 54). Three experiments on different occasions were conducted using the same cell source. Cells were plated in triplicate on 12-well cell culture plates and grown to 70-80% confluence. Optimal LPS/LTA dose and exposure times were experimentally determined according to cell viability and cytokine TNF- α mRNA expression levels. Cells treated with 1 μ g/mL LPS and LTA for 6 and 12 hours were used in the quantitative RT-PCR assay experiments. Cells treated with 1 μ g/mL LPS and LTA for 24 hours were used for cellular measurements of high energy phosphate compounds and oxygen consumption rate. Cell viability was assessed in 96-well plate format by using Promega CellTiter-Glo[®] Luminescent Cell Viability Assay kit following the manufacturer's instruction. All the experiments were performed in triplicate under three different occasions.

Total mRNA Isolation and Quantitative RT-PCR Analysis. Total mRNA was extracted from MCF12A cells using RNeasy Midi Kits (Qiagen Inc., Mississauga, ON) according to manufacturer instructions. RNA purity and quantity was determined spectrophotometrically by measurement at 260 nm and the OD260/OD280 ratio, respectively, with a UV/VIS spectrophotometer (8453E, Agilent Technologies, Palo Alto, CA). Total RNA was stored at -80°C until analysis. Gene sequences for each transporter were obtained from the National Center for Biotechnology Information GeneBank (260) and specific primers were designed using Primer3 software (261) (Table 10.1). Quantitative RT-PCR (QRT-PCR) analysis was carried out using a QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7300 Real-Time PCR system. The QRT-PCR protocol was carried out according to manufacturer's instructions. The protocol consisted of reverse transcription (1 cycle at 48°C, 30 minutes), PCR initial activation step (1 cycle at 95°C, 15 minutes), three-step thermal-cycling (50 cycles; denaturing at 94°C, 15 seconds, annealing at 60°C, 30 seconds, and primer extension at 60°C for 30 seconds), and a melt curve analysis from 65°C-95°C at 0.5°C/second.

Validation of the $2^{-\Delta\Delta CT}$ Method. Real-time PCR assays were initially optimized to give PCR efficiency between 1.9-2.1 (as determined by a 3-point standard curving using serial dilutions of control RNA with a slope range of -2.9 to -3.5) and a single

melt-peak corresponding to the appropriate PCR product as verified by 2% agarose gel electrophoresis. The reactions were further optimized for usage of the $2^{-\Delta\Delta C_T}$ method using β -actin as an internal standard. The amplification efficiency of each target and β -actin was determined by constructing a standard curve from C_T and RNA concentration. The target genes and β -actin were then amplified using same diluted samples. The ΔC_T were calculated (i.e. the difference between the target gene C_T and β -actin C_T). The slope from log RNA concentration versus The ΔC_T was close to zero (<0.1). Only primers giving PCR amplification close to 100% and the relative efficiencies between the target and β -actin that were approximately equal were used in our experiment. Fold differences in mRNA expression between control and treated samples were then calculated.

High Energy Phosphate Compound Measurement. High-energy phosphate substrate levels including ATP, ADP and AMP were measured with HPLC-UV method as described by Olkowski et al., (285). Briefly, MCF12A cells were quickly washed with 1 mL PBS twice after a 24 h exposure to LPS, LTA, or vehicle. To each well, 0.35 mL of 0.7 M ice cold perchloric acid (MW 100.46) was added and cells were detached from the plate by using a cell scrubber. The mixture was then homogenized and centrifuged at 12,000 rpm in an Eppendorf microcentrifuge (Model 5417 C, Brinkman instruments, Westbury, NY, USA) for 5 minutes. The supernatant was collected and neutralized with 2 M potassium hydroxide to bring the pH to 7.0. The supernatant was then filtered through 0.45 μ m filter (ChromSpec, Brockville, Ontario, Canada) and 10 μ L was injected onto a HPLC analytical column (150 \times 4.5 mm, 3 μ Luna ODS3, Phenomenex, Torrance, CA) using a gradient system. The mobile phase was 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer adjusted to pH 7.0 and methanol with the gradient set at 100% phosphate buffer from 0 to 6.5 min, 100% methanol from 6.5–12.5 min, and 100% phosphate buffer from 12.5 to 25 min for column re-equilibration. The mobile phase was delivered at a rate of 1 mL/min and detection was performed at 210 nm. The HPLC system consisted of Agilent 1200 series quaternary pump (G1311A), standard and preparative autosampler (G1329A), and diode array and multiple wavelength detector (G1315D) (Agilent, USA).

Calibration curves were constructed from known concentrations of 0.625 to 20

μg/mL for ATP, 1.56 to 50 μg/mL for ADP and 3.125 to 100 μg/mL for AMP standard solution in blank cell homogenate. The calibration standards were diluted with PBS to achieve calibration standards in a total volume of 1 mL. Three quality control (QC) samples at 16, 4 and 1 μg/mL for ATP, 40, 10 and 2 μg/mL for ADP and 80, 20 and 5 μg/mL for AMP were prepared independent of those used for the calibration curves. The mixtures were extracted using the same method as the samples. Concentration of the high energy phosphate substrates were calculated by interpolation from the linear calibration curve using external standards. Intra- and interday accuracy and precision was <15% for all analytes.

Oxygen Consumption Rate. After 24 h LPS/LTA treatment, cell viability was assessed by Trypan blue exclusion and MCF12A cells were re-suspended in freshly prepared growth media at 5×10^6 cells/mL. The oxygen consumption rates were measured at 37°C using the Hansatech oxygen electrode (Hansatech Instruments Ltd, UK) with cell free media as blank. Oxygen consumption rates were obtained by measuring the oxygen concentration in a closed chamber over time and finding the slope that resulted in a linear plot. Oxygen consumption rates were expressed as nmole/min/ 10^6 cells.

Statistics. All data are reported as Mean \pm SEM. All experimental data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The mRNA expression levels of various transporters after LPS or LTA treatment were compared to control using unpaired t-test. Multiple comparisons for fold difference caused by LPS or LTA with different incubation time for each target gene were analyzed using one way ANOVA with Tukey's Multiple Comparison Test. Means of ATP, ADP, AMP concentrations and oxygen consumption rate in MCF12A cells treated with LPS or LTA were compared separately with control using paired t-test. Significance level was set at $\alpha = 0.05$.

10.4 Results

To determine a noncytotoxic concentration of LPS and LTA, MCF12A cells were incubated with increasing concentrations of LPS/LTA for 24 hours. LPS caused a concentration-dependent decrease in MCF12A viability, while Lipoteichoic acid (LTA)

had limited effects (Figure 10.1). Cell viability remained >95 % at 1 $\mu\text{g/mL}$ LPS and LTA, and this concentration was used for all remaining experiments. TNF- α mRNA expression levels were assessed to ensure that 1 $\mu\text{g/mL}$ LPS and LTA was sufficient to elicit an immune response in MCF12A cells. LPS stimulated a rapid increase in TNF- α expression with expression levels highest at 6 h and declined by 12 h exposure (Table 10.2). On the other hand, LTA induced a delayed response with TNF- α expression levels significantly higher than control only after 12 h duration of exposure (Table 10.2).

Both LPS and LTA caused time-dependent changes in mRNA expression levels of selected transporters (Table 10.2). LPS significantly downregulated the mRNA expression of OCTN1, OCTN2, FATP1, and FABP3 relative to control, but upregulated GLUT1, GLUT8 and SGLT1 in MCF12A cells after 6 hours of incubation ($P<0.05$). Transporter expression generally returned to control levels or exceeded control (OCTN2, GLUT1, FATP1, and FABP3) after 12 hour incubation ($P<0.05$). Incubation with LPS for 6 or 12 h had minimal effect on the expression levels of FATP4. LTA caused limited changes in transporter expression after 6 hours of incubation except for slight decreases in GLUT1, GLUT8 and SGLT1 expression. After 12 hours of exposure, LTA significantly downregulated the mRNA expression levels of GLUT1, GLUT8 and SGLT1 but upregulated OCTN1, FATP1 and FABP3 expression ($P<0.05$).

Furthermore, the fold differences in transporter mRNA expression levels caused by LPS or LTA treatment were compared (Table 10.2). After 6 hours of incubation, the LPS-induced changes in OCTN1, GLUT1, GLUT8, SGLT1 and FATP1 expression levels were significantly different from LTA ($P<0.05$). At 12 h, the FD in OCTN2, GLUT1 and GLUT8 expression caused by LPS were significantly different from LTA treatment ($P<0.05$).

To determine whether altered transporter expression levels with LPS and LTA treatment altered energy balance in MCF12A cells, the concentration of high energy phosphate substrates, ATP, ADP and AMP, and the oxygen consumption rates (OCR) in MCF12A cells after 24 hours LPS or LTA exposure were measured. Both LPS and LTA failed to change ATP, ADP, and AMP levels in the MCF12A cells. However, LTA caused a significant decrease in OCR in MCF12A cells ($P<0.05$) (Figure 10.2).

Table 10.1. Primer sequences for quantitative RT-PCR of L-carnitine, glucose, and fatty acid transporters in MCF12A cells.

Gene	Accession Number	Primer Sequences	
		Forward	Reverse
OCTN1	NM_003059	ggcgcaacaacagtgtcc	ctgaactcccagccatcc
OCTN2	AB015050	Gtcacggctgcctttcc	ggatggctgtcaggatgg
GLUT1	NM_006516	accgcaacgaggagaacc	ccacagcgatgaggatgg
GLUT8	NM_014580	Cactcagcttcggcttcg	acggagcacagcaagagg
SGLT1	NM_000343	gcgctaaggccctgatctt	ggcagtagaggcctcctca
FATP1	NM_198580	cactcggtgtgggttttgc	tgtgtgttttaggggtgagaatgc
FABP3	NM_004102	Gtgtgggaatcacgatgg	ctgtgctcacacgacagc
FATP4	NM_005094	Catgcggcccaacga	cgattcccacgatgttctct
TNF α	NM_000594	Cggtgcttgttcctcagc	ctgtgcactcgggggttcg

FABP, Fatty Acid Binding Protein; FATP, Fatty Acid Transport Protein; GLUT, Sodium-Independent Glucose Transporter; OCTN, Organic Cation/Carnitine Transporter; SGLT, Sodium-Dependent Glucose Transporter 1; TNF α , Tissue Necrosis Factor-*Alpha*.

Table 10.2. Mean \pm SEM fold difference relative to control in mRNA expression levels of various transporters in MCF12A cells incubated with 1 μ g/mL lipopolysaccharide (LPS) or Lipotechoic Acid (LTA) for 6 or 12 hours (n=3).

Target Gene	LPS		LTA	
	6 h	12 h	6 h	12 h
TNF-alpha	1.54 \pm 0.12 ^{a*}	1.17 \pm 0.19 ^{ac}	0.83 \pm 0.09 ^{bc}	1.70 \pm 0.08 ^{aξ}
OCTN1	0.50 \pm 0.09 ^{a*}	1.18 \pm 0.12 ^{bc}	0.82 \pm 0.06 ^{ac}	1.11 \pm 0.04 ^{bc}
OCTN2	0.59 \pm 0.09 ^{a*}	1.95 \pm 0.23 ^{b*}	0.80 \pm 0.04 ^{aξ}	1.11 \pm 0.02 ^{aξ}
GLUT1	1.72 \pm 0.13 ^{a*}	1.60 \pm 0.10 ^{a*}	0.85 \pm 0.04 ^b	0.70 \pm 0.05 ^{bξ}
GLUT8	1.69 \pm 0.07 ^{a*}	1.16 \pm 0.09 ^b	0.79 \pm 0.08 ^c	0.74 \pm 0.05 ^{cξ}
SGLT1	1.44 \pm 0.13 ^{a*}	1.03 \pm 0.11 ^{ac}	0.84 \pm 0.07 ^{bc}	0.76 \pm 0.05 ^{bcξ}
FATP1	0.60 \pm 0.15 ^a	1.37 \pm 0.04 ^{b*}	0.99 \pm 0.02 ^c	1.26 \pm 0.06 ^{bcξ}
FABP3	0.80 \pm 0.13 ^a	1.10 \pm 0.06 ^{ac}	0.94 \pm 0.01 ^{ac}	1.21 \pm 0.08 ^{bcξ}
FATP4	0.99 \pm 0.02 ^a	1.14 \pm 0.02 ^{a*}	1.07 \pm 0.13 ^a	1.22 \pm 0.01 ^{aξ}

Expression was normalized to β -actin and fold difference were determined using the comparative C_T for relative quantitation. Multiple comparisons for fold difference caused by LPS or LTA with different incubation time for each target gene were analyzed using one way ANOVA with Tukey's Multiple Comparison Test. Significance level was set at $\alpha = 0.05$.

^a Means with the different letters are significantly different from each other. Means with *, ξ are significantly different from the control. $\alpha = 0.05$.

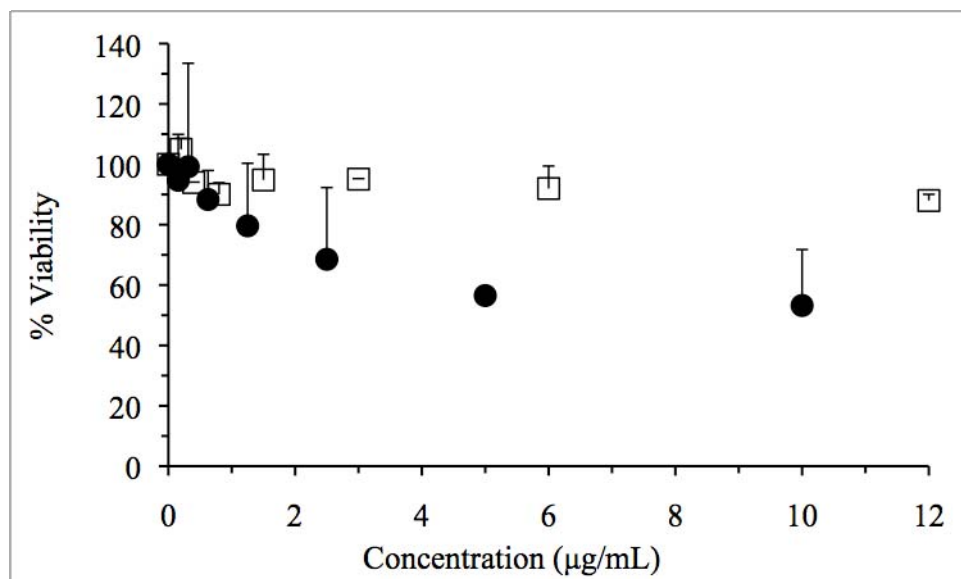


Figure 10.1. Concentration-dependent cell viability of the human immortalized mammary epithelial cell line, MCF12A, to LPS and LTA. MCF12A cells were incubated with different concentrations of LPS (●) /LTA (□) for 24 hours and cell viability determined using Promega CellTiter-Glo® Luminescent Cell Viability Assay kit.

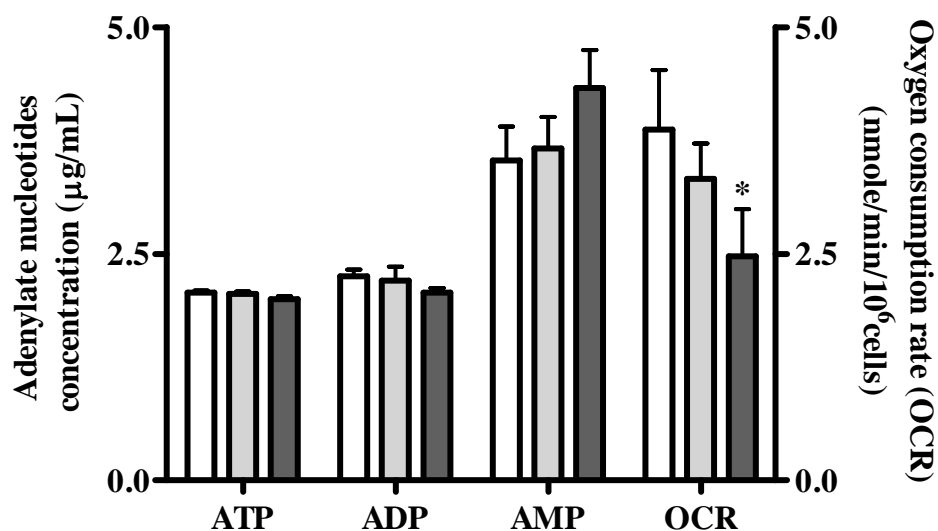


Figure 10.2. Mean (+ SEM) concentrations of ATP, ADP, AMP and oxygen consumption rate in MCF12A cells incubated with vehicle (open bar), 1 µg/mL LPS (light grey bar) or 1 µg/mL LTA (dark grey bar) for 24 hours. Means (n=3) were compared with control using paired t-Test, * $\alpha = 0.05$

10.5 Discussion

A possible factor contributing to the pathogenesis of mastitis may involve the ability of different inflammatory stimuli to alter transporter expression and, hence, substrate availability to lactating mammary epithelial cells. In the present study, we tested the effects of LTA and LPS on glucose, fatty acid and L-carnitine transporter expression levels in the human mammary epithelial cell line, MCF12A, as these transporters make available critical substrates that support energy requirements, biosynthetic capacity and milk composition of various nutrients. Our data suggests inflammatory stimuli alter glucose, fatty acid and L-carnitine transporter expression and cellular metabolism in MCF12A cells. Furthermore, LPS and LTA exposure showed qualitative and quantitative differences in the time course of the innate immune response (as assessed through TNF α expression) and in the expression of glucose, fatty acid, and L-carnitine transporters in mammary epithelial cells.

Since gram positive and gram negative bacteria evoke a different innate immune response (137), these differential inflammatory responses may cause dissimilar outcomes on transporter expression. Lipopolysaccharide (LPS) (gram negative) and Lipoteichoic Acid (LTA) (gram positive) are strong inducers of cytokine release and are widely used to model bacterial challenges of mammalian cells. This provided a compelling reason for their use in the present study. Furthermore, the human MCF-12A cell line is an immortalized mammary epithelial cell line with phenotypic characteristics of mammary epithelial cells *in vivo* (328). MCF12A cells also have been used to examine LPS and cytokine-mediated effects on cell function and gene expression (329-331). Given these characteristics, optimization experiments were conducted with MCF12A cells to determine the appropriate physiological concentrations of LPS and LTA that induced TNF- α expression, as a major cytokine of the innate immune response (332-333), without loss of MCF12A cell viability. As expected, concentration-dependent cytotoxicity was observed with LPS but not LTA (137). However, in MCF12A cells LPS and LTA induced a time-dependent increase in TNF α expression where expression was highest early following LPS administration, while LTA caused a delayed increase in TNF α expression (Table 10.2). This was not consistent with Strandberg et al, 2005 who reported that LPS and LTA stimulation of primary bovine mammary epithelial cells

resulted in rapid stimulation of TNF α expression in the first 2-4 h of challenge, but LPS maintained a sustained response while TNF α expression returned to control levels by 8-16 h following LTA challenge (137). Species differences, differences in the concentration of LPS/LTA employed, and cell source (primary versus immortalized) may explain differences in response to LPS and LTA *in vitro* challenge. Nonetheless, the source of the bacterial stimulus influenced the nature of the innate immune response elicited in MCF12A cells, which compelled us to investigate further whether LPS and LTA challenge might lead to differences in the expression profiles of glucose, fatty acid and L-carnitine transporters.

Both LPS and LTA altered glucose, fatty acid and L-carnitine transporter expression in MCF12A cells. Major changes in expression seemed to correspond to the exposure time at which TNF α expression levels were highest. However, the extent and direction of change in transporter expression was different between LPS and LTA suggesting that TNF α alone may not be the principal cytokine regulator of transporter expression. Many cytokines are produced within the mammary gland by mammary epithelial cells and inflammatory cells present or recruited to the gland (334). Cytokines may have differential and/or overlapping effects on transporter expression and may involve different levels of regulation, i.e. transcriptional or post-transcriptional. Although LPS and LTA are considered strong inducers of inflammatory cytokines (335), LPS and LTA could regulate transporter expression through mechanisms that do not involve cytokine release and autocrine regulation.

With regards to the qualitative and quantitative differences in transporter expression levels, most significant is the differential effects of LPS and LTA on glucose transporter expression, where downregulation is observed with LTA but upregulation with LPS. In particular, GLUT1, the principal glucose uptake transporter of the mammary epithelium (336-337), was persistently upregulated following LPS challenge, but downregulated by LTA exposure. GLUT8 and SGLT1 expression returned to control levels with more prolonged exposure (12 h) to LPS and the expression of these transporters remained persistently low with LTA challenge. These expression changes are important to note because glucose functions both as the main energy fuel and

biosynthetic precursor (i.e. for lactose, short-chain fatty acids) in the lactating mammary gland (312). Several studies have identified the ability of inflammation to alter glucose transporter expression with concomitant changes in glucose transport rates (147-148, 324-325). Although the functional outcomes of such expression changes in the mammary epithelium require further investigation, our expression data suggests that chronic LPS and LTA exposure increases and decreases, respectively, the capacity for glucose uptake in the mammary epithelium. Alterations in glucose availability could significantly impact the metabolically active lactating mammary epithelial cell. Whether this contributes to the pathogenesis of mastitis warrants further investigation.

Interestingly, LPS and LTA challenge caused reciprocal changes in the expression of transporters critically involved in fatty acid uptake and/or oxidation relative to their effects on glucose transporter expression. Short-term exposure (6 h) of LPS resulted in the reduced expression of FATP1, FABP3, OCTN1 and OCTN2 (while the glucose transporters exhibited increased expression), with expression either recovering with more prolonged exposure (12 h) or, in the case of OCTN2, FATP1, and FATP4, exceeding expression levels relative to control. With LTA OCTN2, FATP1, FATP4, and FABP3 were significantly upregulated with more prolonged exposure (12 h) while the glucose transporters were significantly downregulated. Altered expression of fatty acid and L-carnitine transporters could result in altered substrate availability (338) with concomitant changes in the extent to which fatty acid oxidation contributes to ATP generation in the mammary epithelium and the availability of fatty acids and L-carnitine in the mammary epithelial cell and breast milk. Recently, our laboratory showed that L-carnitine milk levels directly correlated with the mRNA expression of L-carnitine transporters in the lactating mammary gland (1), supporting the notion that substrate availability is influenced by the magnitude of expression of transporters in the mammary epithelium.

A reciprocal interaction between glucose and fatty acid utilization for cellular metabolism is well described in the literature (339-341). Different cell types tend to have a preference for a particular energy substrate to meet its energy requirements (i.e. glucose versus fatty acid). However, decreased cellular availability of one type of energy substrate can result in an increased usage in the other major energy substrate to maintain

ATP generation in the cell (342-343). Although fatty acid oxidation may contribute only ~ 10% of oxidative metabolism in the mammary gland (308), changes in the balance between glucose and fatty acid oxidation may have negative consequences on overall cell function. The reciprocal changes in transporter expression profiles may suggest that with acute LPS exposure epithelial cells may rely almost completely on glucose oxidation, while LTA challenged mammary epithelial cells may enhance utilization of fatty acids to maintain cellular energy requirements. In either case, altered glucose availability may pose a problem to the lactating mammary epithelial cell as glucose is both a preferred energy source and biosynthetic precursor. Interestingly, we found significant decreases in oxygen consumption rates when MCF12A cells were exposed to LTA but not LPS. Unlike glucose metabolism, ATP production from fatty acids metabolism is oxygen-dependent (307). Hence, enhanced fatty acid oxidation due to increased substrate availability should result in higher oxygen consumption rates. We found no differences in ATP, ADP and AMP levels in cells exposed to LTA and LPS. Why LTA challenge results in decreased oxygen consumption rates with changes in transporter expression profiles that are consistent with greater fatty acid utilization requires further investigation.

With short-term LPS exposure (6 h) upregulation of glucose transporters and downregulation of L-carnitine and fatty acid transporters suggests that cellular energy requirements more heavily rely on glucose oxidation. Expression of FATP1 and FATP4, which are responsible for the transport of fatty acids across the plasma membrane, is known to be affected by endotoxaemia (344), with decreased expression reported in various tissues including heart, brain and kidney in patients with sepsis (344). With more prolonged exposure (12 h), GLUT1 remained upregulated but GLUT8, SGLT1, OCTN1, FABP3, and FATP4 returned to normal levels, while OCTN2 and FATP1 were upregulated. Proinflammatory cytokines including TNF- α can increase ATP turnover, requiring a higher rate of ATP synthesis to maintain the cellular ATP pool (345). When a higher rate of ATP synthesis is required, fatty acid rather than glucose oxidation is preferred (307). Indeed, fatty acid oxidation is normally considered as the preferred substrate for ATP production during certain inflammatory conditions such as sepsis (346). This may explain why OCTN2 and FATP1 expression increased with prolonged

LPS challenge and why fatty acid and L-carnitine transporter expression was higher in LTA challenged cells. Such shifts in fuel substrate availability and usage may be necessary biochemical adaptations for cells under stress to ensure steady state ATP levels.

In conclusion, our data indicate that inflammatory stimuli alter glucose, fatty acid and L-carnitine transporter expression in mammary epithelial cells. Furthermore, LPS and LTA exposure showed qualitative and quantitative differences in the time course and expression of glucose, fatty acid, and L-carnitine transporters in these cells. Whether changes in transporter expression levels lead to differences in substrate availability (i.e. glucose, fatty acid, L-carnitine) to support cellular energy metabolism and/or cellular synthesis of milk constituents (i.e. lactose, milk fat) is not known and warrants further investigation. The time-dependent changes in transporter expression and the reciprocal regulation of glucose transporters relative to the L-carnitine and fatty acid transporters also warrant investigation. Since the lactating mammary gland is a complex, heterogenous tissue where critical factors such as limitations in substrate availability (i.e. glucose, fatty acid, L-carnitine), immune cell contribution to the inflammatory response, and differences in transporter expression with stage of lactation are not represented in *in vitro* systems, *in vivo* investigations are necessary complements to future proposed *in vitro* studies.

11 Lipopolysaccharide-Induced Inflammation Downregulates Mammary Gland Glucose, Fatty Acid, and L-Carnitine Transporter Expression at Different Lactation Stages

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Under revision with Research in Veterinary Sciences

11.1 Abstract:

Glucose, fatty acids, and L-carnitine are important substrates that support mammary epithelial cell metabolism, biosynthetic capacity, and milk yield and composition. Our study investigated the effects of LPS-induced inflammation on the expression of several glucose, fatty acid, and L-carnitine transporters in the lactating rat mammary gland at different lactation stages. Day 4, 11, and 18 lactating rats (n=3/treatment) were administered LPS (1 mg/kg) or saline by intraperitoneal (IP) injection. Fold differences in the mRNA expression of glucose transporters Glut1, Glut8 and Sglt1, fatty acid transporters Fatp1, Fatp4 and Fabp3, and L-carnitine transporters Octn1, Octn2, and Octn3, were determined using the Comparative C_T method. The mRNA expression levels of all transporters evaluated, except Fatp4 and Octn2, were markedly higher in mammary gland at lactation day 11 compared to lactation day 4. LPS caused a marked decrease in transporter mRNA expression at each lactation stage except for Octn3 and Fatp1, which were markedly increased with LPS administration at lactation day 4, and Sglt1, which was slightly increased at day 11 of lactation. Our results suggest LPS-induced inflammation generally downregulates glucose, fatty acid and L-carnitine transporter expression. Whether such changes lead to reductions in transporter substrate availability to the lactating mammary epithelial cell requires investigation since decreases in the availability of these nutrients may significantly impact mammary epithelial function and milk quality and yield.

Keywords: Inflammation, nutrient transporter, mammary gland, rat

During lactation mammary epithelial metabolic activities increase dramatically to meet the energy requirement for milk synthesis and secretion in the mammary gland. Although glucose contributes significantly to the energy requirement of the lactating mammary gland (113), both glucose and fatty acids are in demand as precursor molecules to facilitate production of high amounts of lactose, oligosaccharides and triglycerides in the milk (84, 115). L-Carnitine is an obligate nutrient required for efficient utilization of fatty acids as energy substrates in the developing neonate (183). Consequently, mammary gland availability of glucose, fatty acids and L-carnitine is essential for milk production and composition due to their crucial roles as energy substrates and/or as milk nutrients or nutrient precursors (112).

Transporters are responsible for the movement of glucose, fatty acids and L-carnitine across the cell membranes of the polarized mammary epithelial barrier. Glucose uptake is mediated by facilitative glucose transporters (Gluts) and secondary active Na⁺/glucose transporters (Sglts) (75). Fatty acid binding proteins (Fabps), fatty acid translocators and fatty acid transport proteins (Fatps) mediate fatty acid uptake into mammary epithelial cells (86). The organic cation/carnitine (Octn) transporters are responsible for L-carnitine transfer across the epithelium (1, 183).

Inflammatory states are known to alter transporter expression and function in various organs (347). Limited information is available concerning the effects of inflammation on glucose, fatty acid and L-carnitine transporter expression in the lactating mammary gland. Recent studies also show quantitative and qualitative differences in transporter expression between early, middle and late lactation stages (1, 95), and the influence of inflammation on transporter expression could depend on lactation stage. Such differences could have significant implications with respect to milk production and composition, clinical outcomes and therapeutic interventions.

This study represents an initial investigation into the role of inflammatory states on glucose, fatty acid and L-carnitine transporter expression and function at the lactating mammary gland as these transporters make available critical substrates that support the energy requirements, biosynthetic capacity and milk composition of the mammary gland. In this study we challenged lactating rats with lipopolysaccharide (LPS) at early (lactation day 4), mid (lactation day 11) and late (lactation day 18) lactation stages to

determine the effect of LPS on mRNA expression of glucose, fatty acid, and L-carnitine transporters and whether such effects depend upon lactation stage.

Female Sprague-Dawley rats at gestation day 16 were obtained from Charles River Canada (St. Constant, PQ) (n=3 for each lactation stage) and were housed singly in a temperature and humidity controlled facility ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) on a 12-hour light: dark cycle. All rats had free access to food and water. Animal use and procedures were approved by the Animal Care and Supply Committee of the University of Saskatchewan. Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4 serotype) and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Lactating rats received 1 mg/kg (250 μL /kg body weight) of LPS or saline (n=3/treatment) via intraperitoneal (IP) injection and pups were not returned to the dams. Dams were humanely killed at 8 hours post-treatment and mammary glands were rapidly excised, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Total mRNA was extracted from mammary gland using RNeasy Mini Kits (Qiagen Inc., Mississauga, ON) according to manufacturer instructions. RNA purity and quantity was determined spectrophotometrically (UV/VIS spectrophotometer 8453E, Agilent Technologies, Palo Alto, CA) by measurement at 260 nm and the $\text{OD}_{260}/\text{OD}_{280}$, respectively. Gene sequences were obtained from the National Center for Biotechnology Information GeneBank and specific primers were designed using Primer3 software (Table 11.1). Quantitative RT-PCR (QRT-PCR) analysis was carried out using a QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7300 Real-Time PCR system. The QRT-PCR protocol was carried out according to manufacturer's instructions.

QRT-PCR assays were initially optimized to give PCR efficiency between 1.9-2.1 and a single melt-peak corresponding to the appropriate PCR product as verified by 2% agarose gel electrophoresis. The reactions were further optimized for usage of the Comparative C_T method ($2^{-\Delta\Delta C_T}$ method). The amplification efficiency of each target and β -actin (internal standard) was determined by constructing a standard curve from C_T values and RNA concentration. The ΔC_T were calculated (i.e. the difference between the target gene C_T and β -actin C_T) and plotted against the log RNA concentration. Only primers giving PCR amplification close to 100% and the relative efficiencies between

the target and β -actin that were approximately equal (slope <0.1) were used in our experiment. Fold differences were then calculated using the $2^{-\Delta\Delta CT}$ method.

Table 11.2 identifies the effects of LPS on mRNA expression levels of glucose, fatty acid and L-carnitine transporters at lactation day 4 and 11 and the difference in their expression levels in untreated dams between lactation day 4 and 11. Dams at lactation day 18 reached humane intervention points prior to the end of the exposure period requiring their euthanasia. Consequently, no results are available for this lactation stage. The mRNA expression levels of all transporters, except *Fatp4* and *Octn2*, were markedly higher in mammary gland at lactation day 11 compared to day 4 (Table 11.2). LPS administration generally resulted in a marked decrease in transporter expression at each lactation stage except for *Octn3* and *Fatp1*, which were markedly increased with LPS administration at lactation day 4, and *Sglt1*, which was slightly increased at day 11 of lactation (Table 11.2). Our results show that LPS-induced inflammation generally downregulates the mRNA expression of glucose, fatty acid and L-carnitine transporters in both early and mid lactation stages. If changes in expression result in corresponding changes in transporter activity, our results may suggest altered availability of these substrates to the lactating mammary epithelium. Any decrease in the availability of glucose, fatty acids, and L-carnitine may significantly impact mammary epithelial function, biosynthetic capacity, and milk quality and yield.

Glucose transporters previously identified in rodent lactating mammary gland include *Glut1*, *Glut12* and *Sglt1* (97, 348). In our study we detected *Glut8*, a glucose transporter often expressed in tissues under insulin regulation (314), at mRNA expression levels lower than the major facilitative glucose transporter, *Glut1*. Its exact role in the mammary gland, particularly in coordination with *Glut1* and *Sglt1*, is not known. We also observed an increase in glucose transporter expression from early to mid lactation, which is consistent with the literature (85, 97-98). The mammary epithelium relies solely on the maternal circulation to meet its glucose needs (76, 115). As well, regulation of glucose transport in the mammary gland plays a key role in the maintenance of glucose homeostasis in lactation (309). Hence, the increase in glucose transporter expression likely explains the increase in glucose uptake by the mammary gland with the advance of lactation (309) to support the increasing need for lactose

synthesis, ATP generation, and other synthetic capacities. In our study, LPS-induced inflammation decreased glucose transporter expression (with the exception of a small increase in Sglt1 expression at mid lactation), which could have significant consequences on mammary epithelial function and milk yield and quality.

The lactating mammary gland is capable of synthesizing fatty acids and triglycerides *de novo* (76, 115). The *de novo* synthesis of fatty acids and triglycerides depends on extraction of short-chain volatile fatty acids and free fatty acids, respectively, from the blood and the latter process is dependent upon the fatty acid transport proteins examined and the extent of extraction changes with stage of lactation (349). The exact underlying mechanism of fatty acid uptake from maternal circulation is not known but likely involves fatty acid binding proteins and fatty acid transporters (86). We detected Fatp1, Fatp4 and Fabp3 expression in the lactating rat mammary gland. Fatp1 and Fabp3 increased markedly with lactation stage suggesting an important role for these proteins in making available free fatty acids to the lactating mammary epithelium. In our study Octn1 and Octn3 mRNA expression increased with lactation stage though no statistical significance was observed. However, Octn2 expression was similar between early and mid lactation stage, which is not consistent with our previous report where we found Octn2 expression was highest at early lactation and decreased 2-fold in mid-lactation (1). Differences in experimental design (milk was allowed to accumulate for 8 hours in the current study versus 4 hours in the previous study) and limited sample size likely contributed to the differences.

As with the glucose transporters, LPS-induced inflammation generally caused downregulation of fatty acid and L-carnitine transporters irrespective of lactation stage with one exception – at early lactation, LPS administration markedly increased Fatp1 and Octn3 expression. In our previous study, immunohistochemical analysis revealed Octn3 expression in the interstitial space and not the mammary epithelium of the rat lactating mammary gland (1). Octn3 is expressed in the peroxisome and plays a critical role in peroxisomal β -oxidation (202). Upregulation of Fatp1 and Octn3 might suggest enhanced peroxisomal β -oxidation as an important response of the lactating mammary gland to inflammation in early lactation. This finding warrants further investigation.

In summary, LPS-induced inflammation downregulated the mRNA expression of

glucose, fatty acid and L-carnitine transporters in rat mammary gland at early and mid-lactation stages. Whether such downregulation is associated with changes in mammary epithelial cell availability of these substrates requires investigation. Furthermore, the reduction in milk yield and quality associated with inflammatory states such as mastitis could be, in part, due to downregulation of glucose, fatty acids and L-carnitine transporters and this warrants investigation.

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Table 11.1. Primer sequences for quantitative RT-PCR of transporters in rat lactating mammary gland.

Gene	Accession Number	Primers		Product Size
		Forward	Reverse	
Octn1	NM_022270	catggctgtgcagactgg	gcaccatgtagccgatgg	209
Octn2	NM_019269	ggcgcaaccacagtatcc	ggggctttccagtcaccc	244
Octn3	NM_019723	gacaccgtgaacctgagc	ccatccaggcagttctcc	188
Glut1	NM_138827	agctgccctggatgtccta	gagaagccagccacagcaa	151
Glut8	NM_053494	cgcagagcctgctgatgtt	cggtagccacacccttgatg	152
Sglt1	NM_013033	gatgcacccatccagtcc	cagccccacaaagtgacc	222
Fatp1	NM_053580	aggagtggaggggaaagc	tgtagccgggtcttctgg	180
Fabp3	NM_024162	aggagtggaggggaaagc	tgtagccgggtcttctgg	231
Fatp4	XM_231115	tggatgagctgggctacc	gttgctagtggggcttgc	200

Fatp, Fatty acid transport protein; Fabp, Fatty acid binding protein; Glut, Na⁺-Independent glucose transporter; Octn, Organic cation/carnitine transporter; Sglt, Na⁺-Dependent glucose transporter.

Table 11.2. Fold differences in mRNA expression of LPS treated relative to saline administration of glucose, fatty acid and carnitine transporters in Sprague-Dawley dams at lactation day 4 and 11. Fold differences between lactation day 11 and day 4 of saline administered dams are also presented.

Lactation Day	Glut1	Glut8	Sglt1	Fatp1	Fabp3	Fatp4	Octn1	Octn2	Octn3
L4	0.27	1.01	0.39	5.23	0.11	0.57	0.74	0.39	1.77
L11	0.10	0.29	1.51	0.15	0.03	0.60	0.37	0.24	0.45
Between L4 & L11*	10.6	5.02	2.63	5.49	4.55	1.11	5.07	0.95	3.13

Fold differences were calculated according to the $2^{-\Delta\Delta CT}$ method. *Control (saline administered) dams

Table 11.3. Mean \pm SEM mRNA expression levels of glucose, fatty acid and carnitine transporters in whole mammary gland tissue from Sprague-Dawley dams treated with saline (Control) or 1 mg/kg body weight LPS (Treated) at lactation day 4 and day 11 (n=3/treatment; rats on lactation day 4 are different from lactation day 11).

Transporters	Lactation Day 4		Lactation Day11	
	Control	Treated	Control	Treated
Glut1	0.51 \pm 0.13	0.15 \pm 0.02 [*]	6.52 \pm 0.28 ^a	0.38 \pm 0.19 [*]
Glut8	0.40 \pm 0.05	0.33 \pm 0.01	2.47 \pm 0.68 ^a	0.45 \pm 0.08 [*]
Sgl1	3.72 \pm 1.91	1.46 \pm 0.73	8.76 \pm 2.07	15.44 \pm 4.41
Fatp1	0.14 \pm 0.06	0.88 \pm 0.24 [*]	1.05 \pm 0.44	0.14 \pm 0.08
Fabp3	1.07 \pm 0.04	0.15 \pm 0.10 [*]	5.55 \pm 1.31 ^a	0.16 \pm 0.05 [*]
Fatp4	4.18 \pm 1.73	2.10 \pm 0.52	5.23 \pm 2.80	5.30 \pm 2.92
Octn1	0.09 \pm 0.03	0.11 \pm 0.07	0.56 \pm 0.26	0.24 \pm 0.14
Octn2	0.56 \pm 0.06	0.22 \pm 0.05 [*]	0.53 \pm 0.09	0.12 \pm 0.01 [*]
Octn3	0.03 \pm 0.01	0.05 \pm 0.01	0.13 \pm 0.05	0.04 \pm 0.01

Expression was normalized to β -actin. Means between control and treated dams within a lactation stage, or between the control dams at L4 and L11, were compared using unpaired t-test.

^a Significantly different from control at lactation day 4 for a given transporter, P<0.05.

* Significantly different from control within a lactation stage, P<0.05.

12 General Conclusions and Future Directions

12.1 General Conclusions

Exclusive breastfeeding for the first six months of life is highly recommended by a number of health organizations including Health Canada and WHO (5). However, maternal medications used to treat disease in mothers during breastfeeding raise significant safety concerns. Most research in this area has focused on identifying the amount and mechanism of drug transfer into breast milk and the exposure risk to the nursing neonate (102, 152, 156, 223). However, limited information is available on the potential effect of maternal disease and medications on nutrient transporters in the mammary gland and the nursing neonate. Limited research recognizes the potential for disease/drug-nutrient transporter interactions in the lactating mammary gland and the potential of such interactions to affect the levels of specific nutrients and drugs in the breast milk. Similarly, it is generally poorly acknowledged that disease- or drug-nutrient transport interactions can occur in the nursing neonate when the drug is delivered via breast milk. Moreover, it should be understood that the expression levels of transporters in the mammary gland and neonate change with stage of lactation and age of the neonate, respectively, and that these interactions may have different effects depending on the timing and duration of drug administration and or development of disease. My dissertation research offers important advancements in our understanding of the possible interactions between maternal inflammatory disease or drug use and nutrient transport in the nursing mother-neonate dyad. The competitive interaction between the conditionally essential nutrient, L-carnitine, and the beta-lactam antibiotic, cefepime, were used as proof-of-concept in the lactating dam-neonate rat model.

Numerous transporters are expressed in the lactating mammary gland that function to transport nutrients, bioactive molecules and drugs into the breast milk. Substrate specificity of such transporters can include endogenous molecules and drugs. Consequently, a drug and nutrient competing for the same transporter may result in reduced nutrient transport to breast milk. In my study, we identified a significant interaction between a critical nutrient, L-carnitine, and a drug, cefepime, which competes for the same nutrient transporter in the lactating mammary gland. The results indicated that cefepime reduced L-carnitine transfer into rat milk, but most significantly,

this interaction occurred only during early lactation. This study demonstrated that both L-carnitine and cefepime levels in rat milk were found to be higher during early lactation stage compared to mid lactation stage and these levels seemed to correlate with the higher expression levels of Octn2, a major L-carnitine uptake transporter in the rat lactating mammary gland, in early lactation. The ability of a drug to affect the milk composition of a critical nutrient via competitive inhibition of a transporter and the dependence of lactation stage on the significance of the interaction represent highly novel outcomes of my dissertation research. My work suggests that in addition to the amount of drug transfer into milk, the influence of the drug on mammary gland function (i.e. nutrient transporter function) is an additional critical determinant of neonatal exposure risk when nursing mothers require pharmacological interventions. Furthermore, assessment of the possible risk must take into consideration the stage of lactation when drug therapy is instituted as the potential for significant drug-nutrient transporter interactions may change with lactation stage.

In addition to a potential for drug-nutrient transport interactions in the lactating mammary gland, carrier-mediated transport of drugs into breast milk requires additional considerations as drug levels in the milk can change with lactation stage. My dissertation work showed that the milk-to-serum ratio of cefepime changed considerably with lactation stage. Since the milk-to-serum ratio is an important determinant of neonatal exposure indices, any change in the ratio with lactation stage may significantly impact the magnitude of infant exposure risks following maternal medication use during breastfeeding and the assessment of risk. The risk to the neonate is further compounded by the immature drug elimination mechanisms of early postnatal life. The results of my pharmacokinetic study provided experimental evidence for age-related changes in the elimination capacity of cefepime. The higher milk levels of cefepime at earlier lactation stage (high M/S ratio) coupled with the lower capacity to eliminate cefepime at earlier postnatal life resulted in considerably higher values for the exposure indices used to assess neonatal risk to drugs present in the breast milk. My dissertation work clearly identifies the importance of considering both lactation stage and the developmental stage of the neonate when assessing the exposure risk via breast milk drug exposures.

As with the lactating mammary gland, when transporters are shared by a drug and nutrient a possibility exists of a drug-nutrient interaction when neonatal patients require drug therapy. Such processes also undergo ontogenesis during postnatal life. Thus, the magnitude and significance of drug-nutrient transport interaction in the neonatal population could vary depending on timing and/or duration of exposure relative to postnatal maturation. In my study, I identified that cefepime exposure during early life affected L-carnitine homeostasis mechanisms in rat pups, which did indeed depend on the time and duration of the exposure. Neonates demonstrated a capacity to adapt to alterations in nutrient availability by altering the ontogenesis of several L-carnitine homeostasis mechanisms. However, these adaptations were not sufficient in young neonates as evidenced by severe histopathological changes in cardiac ventricular tissue. These findings could have major implications with respect to drug treatment of neonates highlighting a potential need for nutritional interventions during drug therapy in this population.

Inflammation of the mammary gland is a very common condition during breastfeeding. Presently with the majority of research being focused on the immunological factors that lead to pathogenesis and recovery of the mammary gland from infection, I investigated the effects of different inflammatory stimuli on the expression of energy substrate transporters in mammary gland. Alterations in energy substrate availability and utilization may explain, in part, the pathogenesis of mastitis and of other diseases in which epithelial barrier integrity is a critical underlying factor in the disease process. In vitro studies with a human mammary gland cell line showed that two widely used inflammatory stimuli, one gram-negative and one gram-positive, altered the expression of glucose, fatty acid and L-carnitine transporters in a time-dependent manner. The extent and direction of change in transporter expression was found to be different between the two bacterial stimuli. However, whether such differences contribute to the different pathological manifestations of mastitis caused by gram-negative and gram-positive bacterial stimuli was not investigated and requires further studies. Interestingly, the research demonstrated reciprocal regulation of glucose transporters relative to the L-carnitine and fatty acid transporters. This may suggest that with gram-negative inflammatory stimuli mammary epithelial cells may rely almost

completely on glucose oxidation (upregulated glucose transporters), while gram-positive bacterial stimuli challenged mammary epithelial cells may enhance utilization of fatty acids to maintain cellular energy requirements (upregulated fatty acid transporters). Additionally, my *in vivo* study indentified that a gram-negative bacterial inflammatory stimulus markedly reduced expression of glucose, fatty acid and L-carnitine transporters in rat mammary gland at early and mid lactation stages. However, knowledge of whether changes in transporter expression levels lead to differences in substrate availability (i.e. glucose, fatty acid, L-carnitine) to support cellular energy metabolism and/or cellular synthesis of milk constituents (i.e. lactose, milk fat) was not obtained and thus further investigations are needed to explore this possibility. Nonetheless, these studies provide experimental evidence to support my novel hypothesis that inflammation may affect epithelial barrier function through alterations in the expression of energy substrate transporters.

Collectively, the results of my research provided experimental evidence for significant disease- or drug-nutrient transport interactions in the nursing mother-neonate dyad. In a practical sense, my research offers invaluable information to health care professionals in their pharmacological management of the nursing mother-neonate dyad. My research may one day point to a need for dietary modification or supplementation when medications are administered to the nursing mother-neonate dyad to minimize the risk for unfavourable outcomes following a significant drug-nutrient transport interaction. Moreover, the results of my research identify the possible risks associated with maternal disease that may consequently alter mammary gland function and milk composition. Additional research in this area may lead to the identification of improved nutritional intervention strategies for mothers with certain diseases, such as inflammation, who still wish to breastfeed, but do not wish to place their nursing child at risk.

Although drug administration to the nursing mother or the neonate is necessary to reduce the morbidity and mortality associated with disease, drug use is not without its own risks, particularly in the nursing mother-neonate dyad. My research represents the acknowledgement of one possible significant risk factor associated with drug use in this population. My dissertation research has made some considerable first steps towards an

understanding of this risk, but much more research is needed into this novel area of investigation.

12.2 Future Directions

My work provided experimental support of a significant *in vivo* drug-nutrient transport interaction in the nursing mother-neonate dyad. Identification of this interaction leads to a number of possible additional investigations. In the neonate, my work suggested that the interaction resulted in changes in nutrient homeostasis pathways depending on timing and duration of drug exposure relative to postnatal age. However, whether these changes are permanent or transient are unknown. Future studies are needed to investigate the potential for metabolic programming of nutrient homeostasis pathways following a drug-nutrient transport interaction and the possible long-term consequences of such interactions on the risk for adult onset disease.

In the lactating mother, future studies could investigate other relevant drug-nutrient transport interactions at the lactating mammary gland and the health consequences of such interactions on the breastfeeding mother-infant dyad. Furthermore, studies are needed to determine whether alterations in the milk levels of specific nutrients resulting from a drug-nutrient transport interaction at the lactating mammary gland can significantly impact nutrient availability and the ontogenesis of nutrient homeostasis pathways in the nursing neonate. Such studies are critically important in determining whether nutritional interventions are required to overcome nutrient deficiencies in the breastfeeding mother-infant dyad imposed by an interacting maternal medication and/or disease.

Maternal disease-nutrient transporter interactions were also identified in the mammary gland at transcriptional levels. Future studies are needed to investigate whether changes in energy substrate transporter expression levels eventually lead to alterations in cellular energy metabolism with consequent effects on milk yield and quality. Although the focus of my work was on the mammary gland, disease-nutrient transporter interactions can also occur in other epithelial barriers. Future investigations into whether changes in energy substrate transporter expression profiles could contribute to diseases associated with other epithelial barriers are warranted.

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APPENDIX A

Supplemental Table 1. Primer sequences for quantitative RT-PCR of L-carnitine transporters in whole rat lactating mammary gland.

Gene	Accession	Primers		Control
	Number	Forward	Reverse	Tissue
Octn1	NM_022270	catggctgtgcagactgg	gcacatgtagccgatgg	Kidney
Octn2	NM_019269	ggcgcaaccacagtatcc	ggggctttccagtcaccc	Kidney
Octn3	NM_019723	gacaccgtgaacctgagc	ccatccaggcagttctcc	Testis
Atb ^{0,+}	AF_320226	gtgtgggaatcacgatgg	ctgtgctcacacgacagc	Lung
Ct2	BC_100473	cagtccttgcttcgatgg	agaagaacacgccgatgc	Testis

Octn1, organic cation/carnitine transporter 1; Octn2, organic cation/carnitine transporter 2; Octn3, organic cation/carnitine transporter 3; Atb^{0,+}, amino acid transporter system B^{0,+}; CT2, L-carnitine transporter 2.